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Analysts Of The Organic Acids Of Bryophyllum Calycinum

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ANALYSIS OF THE ORGANIC ACIDS OF *BRYOPHYLLUM CALYCINUM*

by

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ABSTRACT

The organic acids present in Bryophyllum calycinum leaf tissue were characterized and identified by chromatography and infrared spectroscopy. The amounts of these acids were determined by gas chromatography. The major acids present were malic, citric, and isocitric acids. Malic and isocitric acids each composed approximately 38% of the total acid content and citric acid made up 12%. The remaining large percentage was accounted for by minor acids which included lactic, oxalic, glyoxylic, fumaric, pyruvic, succinic, oxalacetic, α-ketoglutaric, and cis-aconitic acids. Fumaric, pyruvic, and succinic acids each represented about 1% of the total acid content and cis-aconitic acid composed 1.5%. In addition, a large number of minor acids were tentatively characterized but not identified. These were mono-, di-, and tricarboxylic acids apparently containing from three to seven carbons in the carbon chain. Many of these acids evidently had functional groups, including hydroxyl groups, in addition to their carboxyl groups. The boiling points of the methyl esters of these acids ranged from about 140°C to 310°C. These unidentified minor acids were present in amounts ranging from less than 0.1% to 1%.

Extraction of the organic acids from dried leaf tissue with water containing H^+ form resin gave reproducible results. Minor

acids could be completely separated from each other and from major acids only after a series of chromatographic procedures, which exploited different chemical characteristics of the acid molecules, had been employed. The separation and determination of acids present in amounts ranging from 50% to 0.025% of the total acid content was possible using as little as 0.1 gram of dried leaf tissue. Purification and fractionation of the plant extract on carbonate form anion exchange resin allowed complete recovery of acids and provided a good initial separation. Cellulose was superior to silica gel for thin-layer separation of extracted acids if the acids were to be eluted but silica gel provided sensitive analysis of separated acids. Acids of Bryophyllum which had been quantitatively recovered from cellulose and known acids were esterified at elevated temperature in methanol containing H^+ form cation exchange resin. Esterification and recovery of standard acids, with the exception of oxalic acid, was complete but side reactions occurred with keto acids. A column containing Carbowax 20 M provided selective separation of the methyl esters in gas chromatography. Preparative ester separation was carried out on SE-30 silicone and pure ester samples were collected for which infrared spectra were obtained. Comparison of these spectra with the spectra of known esters allowed identification of acids from the extract and gave information as to the structure of unidentified acids. The identity of these acids could evidently be determined by comparing their spectra with the spectra of the esters of known acids whose chromatographic behaviour and structural features matched those of the unknown acid.

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CHAPTER I

INTRODUCTION

1. ORGANIC ACIDS

A. General

The organic acids of Bryophyllum calycinum were analyzed so that previously uncharacterized acids present in the leaf tissue could be characterized and identified. The amounts of these acids present were determined. B. calycinum was chosen for this study since its acid metabolism has been extensively examined and since it, like other Crassulaceae, has a high percentage of uncharacterized acids. The relation of the uncharacterized acids to the acid metabolism of the plant is not known. Some of these acids may be involved with crassulacean acid metabolism.

B. Crassulacean Acid Metabolism

Plant cells contain a wide variety of organic acids (14, 105) and specific organic acids are often accumulated in the vacuole (79,p.37). Crassulacean acid metabolism is a special form of acid accumulation. The term refers to the diurnal variation in acidity exhibited by some succulent members of the Crassulaceae in their green parts. During darkness the acid content of the leaf increases but this increase disappears during a subsequent light period. Experiments with intact

plants and excised leaves have shown that changes in the amount of malic acid account for the major part of the diurnal variation in acidity. The changes in the amount of citric acid are smaller than those of malic acid but occur in a similar direction. The large amount of isocitric acid which is present remains constant. The amounts of organic acids present in B. calycinum leaf tissue are listed in Table I.

The increase in the amount of malic acid is due primarily to the fixation of carbon dioxide (9,59,84,92,97,109). The results of Bradbeer et al.(10) indicate that the synthesis of malic acid occurs as follows: ribulose diphosphate, by the action of ribulose diphosphate carboxylase and CO_2, gives two phosphoglyceric acid molecules. These are converted into phosphoenolpyruvate which, in the presence of phosphoenolpyruvate carboxylase and CO_2, is carboxylated to yield oxalacetic acid. Malic dehydrogenase reduces the oxalacetic acid to give malic acid.

The decrease in amount of acid appears to be the result of an increase in the reactions leading to the breakdown of malic acid. In an excess of CO_2, succulent leaves produce acids during exposure to light(94). The experiments of Wood (109) indicate that a competition for CO_2 exists between photosynthesis and the process of acid production.

The mechanism by which the accumulation of acid is brought about in these succulent plants has not been determined. MacLennan et al.(57) found that in Bryophyllum not more than 30% of any measured acid and less than 2% of isocitric acid were involved in turnover pools of acid. The remainder was in storage pools, presumably in the vacuole (91).

TABLE I

AMOUNTS OF ORGANIC ACIDS IN BRYOPHYLLUM CALYCINUM

Amount of Acid, μeq/g dry weight of leaf tissue[a]						Reference
Malic	Citric	Isocitric	Oxalic	Succinic	Uncharacterized	
4571.4	1092.9	2164.3				16
657.1	571.4	1271.4	42.9	18.6		15
730.0	370.0	1470.0			90.0	102
1020.0		2040.0[b]			130.0	c
710.0	249.0	1329.0			246.0	107
428.6 - 3571.4	714.3	2142.9			1571.4 - 2142.9	99
857.1 - 4285.7	357.1 - 928.6	1857.1			2142.9 - 2571.4	101
428.6	142.9	2857.1			2428.6	100
426.1 - 2769.9	223.0 - 557.6	2230.7 - 3346.1				74

TABLE I (Continued)

Amount of Acid, μeq/g dry weight of leaf tissue[a]						Reference
Malic	Citric	Isocitric	Oxalic	Succinic	Uncharacterized	
571.4 - 3714.3	285.7 - 857.1	2571.4	285.7		857.1 - 785.7	75
357.1 - 2857.1	142.9 - 714.3	2357.1			2000.0 - 1071.4	98
670 - 1910	480 - 610	1590 - 1710				48

[a] Where two values are given the first represents the amount after a light period and the second the amount after a dark period.

[b] Includes citric acid.

[c] Wilson, D. G.; personal communication.

Malate produced by dark fixation is apparently physically separated from the malate actively metabolized in the tricarboxylic acid cycle (53).

Accumulation of acids could involve variation in the activity of the enzymes concerned. No evidence of a diurnal variation in the activity of isolated phosphoenolpyruvate carboxylase or malate dehydrogenase has been obtained (5,p.214:91). Khan and Sanwal (42) found that in Nopalea dejecta the in vitro activity of certain Krebs cycle enzymes was maximum when total acidity was minimum and visa versa. Some of the variation in enzyme activity was apparently due to the cyclical elaboration of inhibitors, possibly acids. Warren (106) found that in certain Crassulaceae an in vivo diurnal variation in the activity of phosphoenolpyruvate carboxylase occurred and suggested that this was caused by an inhibitor, probably a product of the dark fixation system. Two extractable inhibitors were obtained; one had the chromatographic properties of citric acid.

In the Crassulaceae control of the movement of solutes and gases may be involved in acid accumulation. Oxygen availability in Crassulacean leaves apparently limits the rate of respiration (5,p.216:91) and thus may limit the extent to which O_2 requiring reactions can compete with acid synthesis for the products of carbohydrate breakdown. Klein (44) found that there was a restricted movement of solutes and water both into and within leaves of Bryophyllum. This cellular autonomy based on membrane permeability may extend to intracellular membranes and form the basis for segregation of metabolites such as acids. Accumulation of acids in succulent plants may be based on a special control of solute movement across cell membranes involving selective transport systems for specific acids.

C. Uncharacterized Acids

In addition to the major complement of malic, citric, and isocitric acids found in Crassulacean plants, there occur smaller quantities of other acids, some of which have been identified. Vickery and Wilson (102) examined a number of Crassulaceae and found a considerable uncharacterized acid fraction in each. Wilson (107) studied the organic acids in the leaves of B. calycinum by paper chromatography after their initial separation on anion exchange resin. The sequence of known acids eluted from the column was: succinic, malic, citric, isocitric, isocitric lactone, and oxalic. A number of acidic components in addition to these were observed on the paper chromatograms. Some unknown acids were present in the succinic acid fraction and were not completely separated from the earliest fraction containing malic acid. Some unknown acids were present in the transition region between malic and citric acids, in the fractions containing isocitric lactone and oxalic acid, and in fractions immediately before or subsequent to the chloride front. Using the ion exchange method of Palmer (66), the unknown acids in the fractions preceding malic acid plus succinic acid totaled 11.5 mmole per 200 g of dried leaf tissue. There were 13.1 mmole in the citric acid peak. The combined total of 24.6 mmole represented 10% of the total acid content of B. calycinum.

As early as 1942, Pucher (73) stated that the unknown acid fraction of B. calycinum contained oxalic and succinic acids. Krebs and Eggleston (48) found trace amounts of fumaric acid and succinic acid in the leaf tissue of this species while Wolf (108) detected lactic acid in the same tissue. Saltman (85) has obtained evidence that

oxalacetic, α-ketoglutaric, and pyruvic acids are present in B. calycinum leaves. Vickery (98) estimated that there were up to 10 acids in the undetermined acid fraction of this species.

The amounts of the minor acids determined by various authors are presented in Table I. Since in two cases the amount of uncharacterized acid was higher in the dark than in the light and in two other cases the reverse was true, no conclusions can be made with regard to the relation of the change in amount of these acids to crassulacean acid metabolism.

However, labelling of minor acids does occur during the dark fixation of $C^{14}O_2$ by B. calycinum. Varner and Burrel (97) found that the radioactivity of malic acid remained high during 4 hours of darkness. The percentage of radioactivity in succinic acid increased but the specific activity remained the same, approximately 30% that of malic. The specific activity of citric acid decreased from 50% that of malic until it was less than 30% after 4 hours. The specific activities of isocitric and oxalic acids were very low. Saltman (85) determined that the percentage of total radioactivity in succinic acid was highest after 5 minutes and decreased to one half this value on longer exposure. The maximum percentage of total radioactivity found in fumaric acid was almost the same as that in succinic acid but the peak in activity occurred after 10 minutes. After an exposure of the leaves to $C^{14}O_2$ for one minute, no radioactivity was found in either acid. The total percentage of radioactivity present in known acids was less than 100% for all periods of exposure and decreased from 99.4% after one minute to 96-98% after longer periods of exposure. The remaining radioactivity was present in small amounts of unidentified

compounds, possibly acids.

The above discussion has centered on the minor acids of B. calycinum but, as indicated earlier, other members of the Crassulaceae have acids in addition to malic, citric, and isocitric. Fumaric and succinic acids are present in Bryophyllum daigremontianum (19) as well as α-ketoglutaric, pyruvic, aconitic, glycolic, and at least four uncharacterized acids (40). Wilson (107) determined that in Bryophyllum crenatum leaves there were 39.8 mmole of unknown acids, including succinic, in a total acid content of 339.1 mmole. Plants which show crassulacean acid metabolism are found in other families and these plants also have undetermined acids. Nordal et al. (63) discovered at least five acids in addition to malic and citric acids in Opuntia. In Kleinia repens, a number of unidentified acids were present in addition to oxalic, oxalacetic, malic, citric, and pyruvic acids (62).

The origin and metabolic role of the uncharacterized acids found in B. calycinum and other species is unclear. Some acids may be formed as a consequence of crassulacean acid metabolism and some may arise independently. Determination of the identity of these acids may lead to the discovery of the enzymes which produce and utilize them. This in turn should clarify the origin of these acids and enable determination of the part that they play in the metabolism of leaf tissue showing crassulacean acid metabolism. As indicated by Khan and Sanwal (42) and Warren (106), some of these acids may act as enzyme inhibitors and influence the diurnal variation in acidity. Other uncharacterized acids may cause the accumulation of acid to cease by combining with the transport system between the site of synthesis and region of storage of malic acid. However, other acids are probably not involved with crassulacean

acid metabolism in this fashion and may be part of other metabolic systems which may or may not have any connection with the diurnal variation in acidity. Some of these acids may be largely in turnover pools and available for metabolism whereas others may be mainly in storage pools. Although the total content of uncharacterized acids does not show a consistent pattern of diurnal variation, individual acids may accumulate and decrease in amount in a diurnal fashion.

2. ANALYSIS OF ORGANIC ACIDS

A. General

In order to determine the characteristics or identity and the amount of the individual unknown acids, it was necessary to separate these acids from the large amounts of malic, citric, and isocitric acids and then use further chromatographic procedures to separate the minor acids from each other.

B. Extraction

The analysis of the uncharacterized acids depended on the quantitative extraction of organic acids from the plant tissue. Ether extraction of dried plant material has been satisfactorily employed after conversion of the salts of organic acids to the free acids by acidification of the tissue with sulphuric acid (103). Warm water extraction of dry plant material has been used (64,73) but some acid salts may not be satisfactorily removed from the tissue (43).

A convenient means for acidification of tissue in aqueous solution utilizes cation exchange resin. Stafford and Loewus (89) used resin to convert the potassium acid tartrate of grape leaves to tartaric acid, thus enabling quantitative extraction of this acid. Clements (18) acidified an aqueous slurry of fruit peel with cation exchange resin and obtained a recovery of oxalic acid in excess of that previously achieved. This method of acidification was used in the extraction of organic acids from B. calycinum. After removal of the insoluble plant residue and resin by centrifugation and filtration, the extract is immediately ready for the further analysis of the organic acids. As well, basic substances such as amino acids and cations are taken up by the resin so

no preliminary purification is necessary to remove these components from the extract.

C. Ion Exchange Chromatography

In this process organic acids combine with positively charged groups on the ion exchange resin and remain fixed there until they are displaced by an aqueous solution of salt or acid.

The organic acids in the initial extract were separated from sugars and other compounds using anion exchange resin. Initially, a strong salt solution was used to displace the purified organic acids into one fraction. By gradually increasing the strength of the eluant, different acids may be collected in separate fractions; this is the basis of ion exchange chromatography which in a later experiment provided a preliminary fractionation of the organic acids of Bryophyllum as well as a purification of the extract.

Palmer (66) separated organic acids on Dowex 1 formate resin using a gradient of increasing formic acid concentration. Removal of oxalic acid from the resin required elution with 0.1 N HCl. Wang and Mancini (104) used a concave gradient of formic acid concentration to separate 18 of 23 organic acids and emphasized that the homogeneity of peaks obtained must be determined by further chromatographic procedures. Davies et al. (21) separated 94 acids by ion exchange chromatography and found that both structure and acid strength were important in determining the elution of acids.

Kliewer (45) purified extracts of organic acids with anion exchange resin in the carbonate form and eluted the acids with 1.5 N $(NH_4)_2CO_3$. Bryant and Overell (13) found that a preliminary separation

of succinic and malic acids from citric acid could be obtained since the former acids were eluted from carbonate form resin with 0.1 N Na_2CO_3. All acids were quantitatively eluted with a 1 N solution. In the present work anion exchange resin in the carbonate form was found to be advantageous for purification and fractionation of the organic acid extract.

D. Thin-Layer Chromatography

Thin-layer chromatography involves the separation of mixtures of compounds by the passage of solvent over a thin layer of substrate held as a coating on a backing. The substrate may be silica gel, cellulose, or other suitable materials.

Organic acids have been separated on thin-layer plates coated with silica gel. This separation is based essentially on adsorption. The movement of solutes is controlled by the adsorptive capacity of the adsorbent for the solutes and for the solvent, and by the solubility of the solutes in the solvent. Both acidic solvents (4,7,69,70,95) which contain formic or acetic acids and basic solvents (4,11,68) which contain ammonia have been used in these separations. The acids have been detected with alkaline potassium permanganate (69), 2,6-dichlorophenol indophenol(68), ammonium molybdate (95), and bromphenol blue (7) or other similar acid-base indicators.

Cellulose thin-layer plates have also been used with organic acids. Here the technique is based on the partition of solutes between a mobile organic phase (the solvent system) and a stationary aqueous phase (held by the cellulose). Solvents used for the separations may again be acidic (4,31,35,51,60,65,77) or basic (4,22,31,35). The position of the acids on cellulose plates may be determined with 2,7-dichloro--

fluorescein (77), aniline-ribose (35), bromcresol purple (60), and bromphenol blue (22,51) or other suitable acid-base indicators (31).

Analysis of the organic acids in the initial or purified extract of B. calycinum utilized both silica gel and cellulose thin-layer plates. Both acidic and basic solvents were used to provide maximum separation of acids. Cellulose was preferred when the separated acids were to be eluted and analyzed by other procedures.

In one experiment paper chromatography was used in place of cellulose thin-layer chromatography to enable the preparative-scale separation of organic acids. Paper chromatography, like cellulose thin-layer chromatography, is a partition process and similar solvents may be used. The method of Palmer (66), which involves separation of the organic acids with an acidic solvent and their detection with an acid-base indicator, was employed in the present studies.

E. Esterification

The procedure used to separate further the organic acids eluted from thin-layer plates was gas chromatography. However, before the acids can be analyzed by this procedure they must be converted to their esters since organic acids decompose before reaching the temperature of their boiling point and have a low vapour pressure. The methyl esters are the usual compounds prepared for gas chromatography although trimethylsilyl derivatives have been used (37). Methyl esters of organic acids have been prepared by the reaction of the acids with diazomethane (16,25,26, 32,34,41,46,47,49,50,55,56,71,76). Both sulphuric (16,33,56,80) and hydrochloric (29,81,82,83) acids have been used as esterification catalysts in methanol solutions. The Lewis acid boron trifluoride has also been employed as a catalyst for the esterification of organic acids (2,49,50,58).

Although diazomethane will apparently esterify organic acids satisfactorily under suitable conditions (25,32,76), undesired reactions may occur. Maleic and fumaric acids appear to be converted to non-volatile pyrazolines by the reaction of excess diazomethane at the double bond (76). This reaction may also occur with cis-aconitic acid (56). Side reactions may be encountered in the esterification of tartaric, α-ketoglutaric, and glyoxylic acids (16,46,76).

Boron trifluoride also will apparently give complete esterification (2,50,58), although low percentage esterification has been reported for some acids (41,55). Under particular conditions reactions other than esterification may occur with this reagent. Pyruvic acid yielded two compounds after esterification (58), and oxalacetic acid could not be satisfactorily esterified (2). Methoxy-substitution may occur at double bonds since this reaction can take place with unsaturated fatty acids (54).

The mineral acid HCl in methanol gives complete esterification of acids (29,82) but decomposition of keto-acids is encountered (41). Sulphuric acid in methanol gave 84 - 90% esterification of organic acids with the exception of cis-aconitic acid (56). Pyruvic acid yielded two compounds when sulphuric acid was used as catalyst (80). Tartaric acid failed to give a peak when subjected to gas chromatography after esterification (33).

Esterification using H^+ form resin as the acid catalyst instead of a mineral acid has been employed to a limited extent with organic and aromatic acids. Tsuda and Matsumoto (96) esterified organic acids including succinic, maleic, and tartaric with ethyl alcohol in the presence of a sulphonic acid resin. Csuros et al. (20) prepared dibutyl phthalate

with ion exchange resin in butanol, and Bochner et al. (8) esterified salicylic acid in a stirred solution of methanol under reflux with Dowex 50 H^+ as catalyst.

The use of cation exchange resin as an esterification catalyst was found advantageous in the analysis of the organic acids of B. calycinum. With this catalyst, the ester solutions may be taken directly from the reaction mixtures or removed from the resin by filtration and used to determine the percentage esterifications by titration or the composition of the solutions by gas chromatographic analysis. Undesired dilutions which may occur during the removal of homogeneous catalysts are thus avoided. As well, the resin is a mild catalyst and there should be less chance of undesired reactions occurring during esterification. Fieser and Fieser (27,p.371) recommended the use of milder catalysts such as sulphonic acids rather than sulphuric acid or boron trifluoride when sensitive acids such as pyruvic are to be esterified. Csuros et al. (20) also stated that the use of ion exchange resins is favoured where reactants are sensitive to acids.

A disadvantage of resin catalysts is that they produce a slower rate of reaction than other catalysts. Experiments by Csuros et al. (20) with equimolar quantities of resin or sulphuric acid as catalysts indicated that ion exchangers attained the same effects as sulphuric acid but that a longer time was required. The elevated temperature used in the present method of esterification eliminated this disadvantage and allowed a smaller amount of resin to be used as a catalyst.

F. Gas Chromatography

The volatile methyl esters of the organic acids of B. calycinum

prepared by the esterification procedure may be separated by the process of gas chromatography and the amount of each ester present may be determined. In gas chromatography the volatile solutes to be separated are injected, volatilized, and then transported by a carrier gas stream which passes through a heated column containing a stationary liquid phase, usually coated on a porous solid support. The sample components undergo repeated partitions before passing out of the column and through a detector where the amount of each component present is determined. When the compounds to be separated do not differ widely in boiling points, they may be analyzed at a constant column temperature with the lower boiling components emerging first. If there is a wide range of boiling points between compounds, a programmed increase in the temperature of the column is used so that the lower boiling components are well separated and the higher boiling substances are satisfactorily eluted. Where compounds of the same polarity and different boiling points are to be separated a non-polar stationary phase is most satisfactory but if the compounds are to be separated according to their differences in polarity, a polar liquid phase should be used.

The esters of organic acids have been separated on polar liquid phases such as butanediol succinate polyester (41,58), diethylene glycol succinate polyester (24,25,26,49,56,82), Carbowax 20 M polyethylene glycol (24,49,50), and other phases of a similar nature (2,16,29,32,46, 47,55,72,83). Non-polar stationary phases, mainly silicones (41,46,47, 49,55) and Apiezon L (41), have been used less commonly. Constant and programmed temperature regimes have been utilized with both types of columns.

The preference for polar stationary phases is based on the fact

that methyl esters of organic acids often have similar boiling points which makes separation on a non-polar phase difficult. The polar stationary phases can selectively separate methyl esters of a similar boiling point as a consequence of the non-ideal interactions between the polar solutes, with their variety of functional groups, and the polar solvent. The polar stationary phase Carbowax 20 M,used in the present studies shows a high degree of selectivity for methyl esters and other polar compounds as a result of its electron donor properties (12) and its hydrogen bonding ability.

Adsorption of polar methyl esters to the solid support used to hold the stationary phase may occur, especially when a small amount of liquid phase is applied. Treatment of the supports to eliminate the polar sites has been used to avoid this difficulty but Kuksis and Vishwakarma (50) found that recoveries of esters even from treated supports were inferior to those obtained when completely non-polar supports such as Teflon were used. Teflon was used in the present work as the solid support for the low loading of Carbowax 20 M,to prevent loss of organic acid methyl esters through adsorption.

A second difficulty which may be encountered with methyl esters is their reaction with the stationary phase. Dimethyl oxalacetate apparently reacted with butanediol succinate in the experiments of Kellog et al. (41) and no peak was obtained. Kowala et al. (47) proposed that hydrolysis of esters occurred on sodium dodecylbenzene sulphonate. The peak areas for methyl pyruvate, methyl lactate, dimethyl malonate, and dimethyl fumarate decreased as the temperature of a diethylene glycol succinate column increased (25). At temperatures above 100°C, methyl

pyruvate gave three peaks. Decomposition of dimethyl malonate and dimethyl oxalate occurred on a butanediol succinate column (1). The low percentage of Carbowax 20 M used in the analysis of the methyl esters of the organic acids of Bryophyllum allowed separations to be carried out at reasonably low temperatures at which the possibility of side reactions should be reduced.

After their separation on the column the individual methyl esters pass through a detector. The detector used in the analytical studies was the flame ionization detector which is more sensitive than the thermal conductivity detector which was employed during preparative separations. The detector response is recorded graphically and the peak area for a compound is proportional to the amount of that compound detected. The response of the flame ionization detector to organic acid methyl esters has been found to be linear from 0.01 to 10 μg (50,58). The absolute response of the detector to each ester varied with the oxygen content of the ester, being lower with the more oxygenated compounds (50,58).

When gas chromatography is used to determine the identity of the peaks which appear during a separation, the retention volumes of the unknown peaks are compared with those obtained at the same temperature and on the same column for known standard compounds. Coincidence of retention volumes on a single column provides only tentative identification and comparisons using other columns should be carried out to resolve any ambiguities. This method of determining the identity of compounds was employed in the analysis of the methyl esters prepared from the acids of Bryophyllum.

It is often necessary to collect the substances separated by gas chromatography in order to confirm their identity by chemical, chromatographic, or spectroscopic methods. The compound emerging from the exit port of the gas chromatograph is usually collected in a cooled trap. If rapid cooling is used, methyl esters often form aerosols which are lost. The esters were collected during the present studies in narrow bore capillary tubing which passed through a cooling zone (36,38,88).

G. Infrared Spectroscopy

Infrared spectroscopy provides a means for determining the structure of molecules. When a compound is irradiated with successive frequencies of infrared radiation, certain wavelengths are absorbed and these wavelengths correspond to the mechanical vibration frequencies of the molecule. Since specific atomic groups absorb within given wavelength regions, the presence of certain atomic groups in the compound can be determined. The precise frequency at which the group will absorb is dependent on molecular environmental factors such as the mass or electronic characteristics of neighbouring groups.

The sample material may be gaseous, liquid, or solid form. The methyl esters of organic acids were conveniently examined as their chloroform solutions in silver chloride cells. An equal thickness of solvent was placed in the reference beam to compensate for absorption of infrared radiation by the solvent in the sample beam and the resulting spectrum was thus that of the solute.

By comparison of the absorption frequencies on the spectra obtained with the known absorption frequencies of specific atomic groups, a great deal can be determined about the structure of the molecules in

question. Information from other studies when incorporated with such evidence may enable precise determination of the structure of the molecule. Often, however, the identity of the compound can only be determined with certainty by a comparison of the spectrum of the compound in question with the spectra of compounds corresponding to tentative structures proposed for the unknown molecule.

Infrared spectroscopy has previously been used in the study of carboxylic acids and their esters primarily from the point of view of examination of group frequencies and interactions (28,86), and not as a means for identifying unknown carboxylic acids. In their study of carboxylic acids, Childers and Struthers (17) found that the infrared spectra of monobasic acids were very similar except when chain branching was present in the molecule. The similarity was more pronounced between the spectra of various dibasic acids. The spectra of the esters of acids were more specific for infrared characterization than those of the acids. The salts of organic acids were found to give a number of sharp specific bands. Dolinsky and Wilson (23) found that some mono- and dicarboxylic acids gave spectra with few peaks and that some peaks were broad and poorly defined. Bernatek et al. (6) and Nordal and Ogner (61) used infrared spectroscopy in the identification of unknown acids in Agave americana and Euphorbia. Derivatives of these acids including the methyl esters were examined and, in combination with other methods, these studies lead to the identification of these acids. Tadenuma et al. (93) confirmed the existence of certain acids in sake by comparing the spectra of the isolated acids with those of authentic samples of the acids.

H. Combinations of Chromatographic Procedures

Since one chromatographic procedure often fails to resolve

completely the components of a mixture, it is necessary to employ additional chromatographic methods when complete separation of the compounds is required. Ferraz and Relvas (26) separated the Krebs cycle acids of plant material on ion exchange and silica gel columns. Those acids which were not resolved were esterified and separated by gas chromatography. Palmer and Wyman (67) separated the organic acids of banana leaves by ion exchange chromatography and further separated the acids of each fraction by paper chromatography, silica gel column chromatography, or additional anion exchange chromatography. Kuksis and Prioreschi (49) purified the organic acids of plant and animal tissue on ion exchange or silica gel columns. Silica gel thin-layer plates were then used to separate the mixtures of organic acids or their salts. The desired bands of acids were collected and the acids were converted to their methyl esters prior to further separation by gas chromatography. They found that the thin-layer technique was the most satisfactory for the purification and preliminary segregation of the Krebs cycle acid mixture. Janak (39), in what is termed "multidimensional chromatography", emphasized the advantages of a preliminary separation of compounds by thin-layer chromatography and subsequent analysis by gas chromatography. The separation by thin-layer chromatography is independent of the vapour pressure of the substance and depends on the functional groups present. Separation by gas chromatography, however, depends on the carbon number, the molecular weight, and the boiling point of the compound as well as its polarity. The use of such multiple procedures follows the rule that successive separations of mixtures of compounds should exploit different chemical characteristics of the molecules.

In the present work the methods of ion exchange chromatography, thin-layer chromatography, paper chromatography, and gas chromatography were employed in the purification and separation of the organic acids from B. calycinum leaf tissue. In successive experiments separation procedures and modifications were added to those previously used in order to avoid the occurrence of artifacts and enable complete resolution of the individual acids. Difficulties encountered when silica gel thin-layer chromatography was used for the initial separation of the acids in the plant extract led to the use of cellulose thin-layer chromatography for this purpose. Since material was extracted from the cellulose when the acids were eluted, the cellulose was washed before use. Artifacts which apparently resulted from the presence of sugars in the extract were eliminated by ion exchange treatment of the initial extract. Although thin-layer chromatography followed by gas chromatography apparently resulted in complete separation of the acids, it was possible that acids whose esters had the same elution temperature were different. Gradient elution of the acids of the initial extract from the ion exchange column provided a separation of the acids before thin-layer or paper chromatography and gas chromatography, thus ensuring that the acids would be obtained in a pure state for examination by infrared spectroscopy.

CHAPTER II

MATERIALS AND METHODS

A. Preparation of Plant Material

Mature leaves of Bryophyllum calycinum were collected during daylight and were dried at 80°C in a forced-draught oven. The dried leaves were ground in a Wiley mill and stored without desiccation. Numerous samples were blended and this mixture was labelled "Bryophyllum calycinum stock tissue".

B. Extraction of Plant Material

Small amounts (0.1 g) of stock tissue were extracted with deionized water in centrifuge tubes (10 ml capacity) at 30 - 40°C in the presence of thoroughly washed Bio-Rad AG 50 H^+ resin (200 - 400 mesh) which had been dried in vacuo. To each 0.1 g sample of tissue was added 0.3 g of dried resin. Water was added to give a total volume of 5 ml. Rapid stirring was provided by a magnetic stirrer. The extraction was continued for 16 hours or for 39 hours.

The slurry was then centrifuged at 9-10,000 R.P.M. for 10 minutes. The supernatant solution was passed through 0.8 μ pore Millipore filters. Deionized water sufficient to give a loose slurry was added to the residue in the centrifuge tube and extraction of the tissue continued for 20 minutes. Centrifugation and filtration were then repeated. The tissue and resin were then placed on the filter and

washed with deionized water. The filtered supernatants and wash were made up to 10 ml in a volumetric. Two ml aliquots of each extract were titrated with 0.04014 N NaOH in duplicate.

C. Standardization of Sodium Hydroxide Solution

A concentrated solution of sodium hydroxide in deionized water was prepared (1 g NaOH / ml H_2O) and allowed to stand for several days. The solution was filtered and stored in a dark bottle. Eight ml of this concentrate were diluted to 3.8 litres with deionized water and the normality of this solution was determined by titration against 0.1 N KH phthalate using as indicator a 1:3 solution of 0.1% thymol blue in 50% ethanol and 0.1% phenolphthalein in 50% ethanol. The titrations were performed to a pale violet end point. The normality of the sodium hydroxide solution was 0.04014 N.

D. Thin-Layer Chromatography

Glass plates 10 cm by 20 cm were coated in a Camag applicator. Silica gel plates were coated with a hand-blended slurry of 20 g of Camag D-0 silica gel in 45 ml of deionized water at a thickness of 0.3 mm. The plates were then air dried, activated by heating at 110°C for 30 minutes, and then stored in a desiccator.

Cellulose plates were coated at a thickness of 0.5 mm with a machine-blended slurry of 10 g of Avicel microcrystalline cellulose in 43 ml of deionized water. These plates were air dried and then stored without desiccation.

One to two μl of solutions for analysis were spotted on the plate 2.5 cm from the base as separate or contiguous spots. When necessary, repeated applications were made. The sorbent was scraped from the

top and side edges of the plates; the plates were then clamped face to face but were separated by a U-shaped cardboard strip covering the scraped portions and extending to just above the solvent level. The "sandwiched" plates were set upright in the solvent contained in a covered glass tank. After the solvent had travelled upwards to a front that was 10 cm past the origin the plates were removed, separated, and allowed to dry.

The substances on the plates were detected by spraying silica gel plates with an alkaline solution of potassium permanganate and cellulose plates with an acid solution of potassium permanganate.

E. Esterification

Bio-Rad AG 50Wx4 H^+ resin (200-400 mesh) was rinsed with methanol to displace the water. The rinsed resin was heated with excess methanol under reflux for at least one hour. The resin was again rinsed and dried in vacuo at room temperature. The dried resin was stored in vacuo in a desiccator over Linde 13X molecular sieve.

Resin was added to known organic acids which were either dry or dissolved in methanol and were contained in screw-top culture tubes of 8 ml capacity. For each 100 μeq of acid, 156 mg of dried resin were added. Sufficient methanol was added to cover the resin and provide a slight excess. Where larger amounts of methanol were present magnetic stirring was used. Approximately 0.5 ml of methanol was needed for 156 mg of resin. The tubes were tightly closed with Teflon-lined screw caps and were heated for 20 hours at 20 pounds pressure in a portable electric autoclave containing an ethylene glycol : methanol solution (3 : 2 by volume). Temperature was calculated to be 105-110°C.

After the esterification procedure the tubes were removed from the autoclave and allowed to cool. The ester solutions were separated from the resin by filtration and aliquots taken from the known volumes of ester solutions were titrated directly. Other aliquots were titrated after saponification with 0.15 N sodium hydroxide solution at 100°C for 5 minutes and passage through Dowex 50 H^+. Aliquots of the known acid solutions from which the samples for esterification were taken were titrated.

F. Gas Chromatography

i. Analytical

A Varian Aerograph 600B "Hy-Fi" gas chromatograph equipped with a flame ionization detector was used for analytical studies. The nitrogen carrier gas was dried by passage through Linde molecular sieve 13X. The hydrogen for the flame ionization detector was produced by a Varian Aerograph 650 hydrogen generator.

The column used was 1/8" x 5' stainless steel containing 2-3% Carbowax 20M on Teflon 6, 40/60 mesh.

ii. Preparative

A Varian Aerograph A-100 gas chromatograph with a thermal conductivity detector was used for preparative separations with helium as the carrier gas. The sensitivity of the instrument was increased by replacing the 470 ohm resistor with a 1000 ohm resistor.

For both analytical and preparative work a Varian G-10 recorder with a 1 mv scale was used.

The column used was ¼" x 10' stainless steel containing 20% SE-30 on 70/80 mesh Aeropack 30.

Fractions of the effluent from the column of the A-100 gas chromatograph were collected in 0.5 mm inside diameter thick-walled capillary tubes, a U-shaped portion of which was cooled in ethanol and ice.

G. Infrared Spectroscopy

The instrument used was a Beckman Microspec infrared spectrophotometer set at double beam operation, which scanned from a wavelength of 2.5 to 14 microns. The gain setting used was 5 to 6.

The gas chromatographic fraction which had been collected in a capillary tube was dissolved in spectroscopy grade chloroform by passage of a few μl of solvent through the tube. This was done just before the ester was to be examined by infrared spectroscopy. The solution was removed with a syringe and a silver chloride micro-cell (Beckman GC-1) with a 2 μl volume and a 0.1 mm path length was filled with sample.

The samples were analyzed with a chloroform blank in the reference beam. The sample cell was positioned in the carrier so as to give a transmission compared to air of approximately 30% at a wavelength of 25 μ. The reference cell was then positioned in the carrier so that the transmission of the sample cell compared to the reference cell was 90-95%.

H. Separation of Organic Acids of Bryophyllum calycinum Stock Tissue on Silica Gel Thin-Layer Plates

A 0.1 g portion of tissue was extracted and the extract was made to a volume of 10 ml. Two 1 ml aliquots of this extract were titrated, dried in an air stream, and dissolved in a small amount of water.

Ten silica gel plates were spotted with the material from one aliquot and 12 with the material from the second. Five spots of 1 μl were placed on each plate and applications were repeated until all the solution had been spotted. The plates were developed in t-amyl alcohol : acetic acid, 25 : 10. Fresh solvent was used for each set of plates. One plate in each set was sprayed with freshly prepared basic $KMnO_4$ solution prepared by diluting a 2% solution of $KMnO_4$ in 5% aqueous NaOH with four volumes of water. The R_F values of the spots were recorded. Regions of the unsprayed plates which corresponded to areas on the sprayed plates where spots appeared were scraped from the plates and attempts were made to elute the acids from one set of plates with methanol and to elute the acids from the second set of plates with 3N NH_4OH.

I. Cellulose Thin-Layer Chromatography and Gas Chromatography of the Organic Acids of Bryophyllum calycinum

A 0.1 g portion of tissue was extracted and the final volume of the extract was 10 ml measured in a volumetric flask. Two 1 ml aliquots of the extract were titrated, combined, dried in an air stream at 45°C, and dissolved in a small amount of water.

Five spots of 1 μl were placed on each of 16 plates coated with microcrystalline cellulose and the applications were repeated until all the above solution had been spotted. Eight plates were developed in n-butanol : formic acid : water, 25 : 3 : 7; and eight in ethanol : 5N NH_4OH, 80 : 20. One of the plates from each set was sprayed with a 1 : 1 solution of 1% $KMnO_4$ and 1% H_2SO_4. The right hand edge on each of the remaining plates was sprayed so that the acids from one spot could be located.

The unsprayed regions of the plates were divided into bands according to the R_F values of the acids in the sprayed region. The corresponding cellulose bands from a set of plates were scraped into a centrifuge tube and the acids were extracted from the cellulose with 3N NH_4OH. The tubes were centrifuged and the supernatant solution was collected. The extraction procedure was repeated twice. The total supernatant solution for each band was passed through a 0.8μ pore Millipore filter and then air-dried at 48°C in an 8 ml culture tube.

Estimates of the total amount of acids in all corresponding bands and hence in each tube were made from the chromatogram. To each tube resin was added and methanol was then added to cover the resin and provide a slight excess. Esterification of the acids in these solutions was carried out in the autoclave. The esters in these solutions were separated by gas chromatography on the Carbowax column. The retention time of the esters was recorded.

J. Separation of the Organic Acids of Bryophyllum calycinum

A 45 ml quantity of wet Bio-Rex 50W H^+ resin (20-50 mesh) which had been thoroughly washed with water was added to 5 g of Bryophyllum stock tissue contained in a 250 ml stainless steel centrifuge bottle. A magnetic stirring bar and 150 ml of deionized water were then added. The tissue was extracted with stirring for 18 hours at 40°C.

After centrifugation of the bottle at 9,000 R.P.M. for 10 minutes, the supernatant solution was filtered through a 0.8μ Millipore filter. Water was then added to the residue in the bottle to give a total volume of 150 ml. The tissue was extracted for 45 minutes, and centrifugation and filtration were again carried out. Water was again added to the residue and a 10 minute extraction was carried out followed by

centrifugation and filtration. The combined supernatant solutions were made to 500 ml in a volumetric flask. Two 4 ml aliquots of this extract were titrated.

A 30 ml Bio-Rad AG 1 x 8 Cl^- column was converted to the $CO_3^=$ form by passage of 2N Na_2CO_3 through the column until no Cl^- was present in the effluent when tested with $AgNO_3$ and HNO_3. The column was rinsed with 1.5N $(NH_4)_2CO_3$ and then with deionized water. One fifth of the extract (100 ml) was passed slowly through the column. The column was rinsed with deionized water (500 ml); 1.5N $(NH_4)_2CO_3$ (1.4 litres) was passed slowly through the column, and the eluate was collected and dried in vacuo at 60°C in a rotary evaporator. The residue was dissolved in water and made to 10 ml in a volumetric flask. To determine the acid recovered from the column, two 0.4 ml aliquots of the 10 ml of solution were passed through Dowex 50 H^+ columns and were titrated.

Microcrystalline cellulose (320 g) was stirred with a large excess of 5N NH_4OH (1.25 litres). The slurry was centrifuged and the cellulose residue was stirred with water to give a thin slurry which was filtered in a Buchner funnel with Whatman #1 paper. The cellulose residue was thoroughly washed on the filter with distilled water and was dried at 80°C in a forced-draught oven. The cellulose was transferred to a beaker and water was then added to the cellulose until a thick slurry was formed. The cellulose slurry was thoroughly mixed with a magnetic stirrer. Glass plates were coated with the slurry to a thickness of 1 mm. Twelve of these plates were spotted with the purified extract. One μl of solution was applied to 26 spots on each plate, the spots being 3 mm apart. Five applications of 1 μl were made

on each spot. Six plates were developed in n-butanol : formic acid : H_2O, 25 : 3 : 7, and the remaining plates in 95% ethanol : 5N NH_4OH, 20 : 5. The plates were sprayed with acid $KMnO_4$ spray along one edge to locate the acids and the R_F values of the spots were recorded.

The unsprayed portions of the plates were divided into horizontal bands according to these results. The corresponding bands of cellulose were scraped from the plates and were extracted with 3N NH_4OH in a centrifuge tube. The solution was centrifuged and the supernatant solution was collected. The extraction and centrifugation were repeated twice. Each fraction prepared from a set of cellulose bands was air dried at 50°C, dissolved in 3N NH_4OH, passed through a fine sintered glass filter, and again dried. Each of the fractions was dissolved in water and passed through a 1.5 cm x 1 cm Dowex $50H^+$ column. The collected eluate was air-dried at room temperature in an 8 ml culture tube just until the last apparent water was evaporated. Thirty μl of methanol were added to each fraction.

One μl of each solution was spotted on a silica gel plate, in duplicate. One set of plates was developed in t-amyl alcohol : acetic acid, 25 : 10. The second set was developed in ethanol : NH_3 : H_2O, 20 : 3 : 2. The plates were sprayed with basic $KMnO_4$ spray reagent. The spots were marked and R_F values were recorded.

One μl of each solution was titrated, after the addition of water, with 0.001 N NaOH.

Dried resin was added to the solutions in the culture tubes according to the titration results in the proportion 156 mg of resin for 100 μeq of acid. Additional methanol was added to each tube to cover the resin and provide a slight excess. The acids were esterified

using the autoclave. The esters in the esterified fractions were separated by programmed temperature gas chromatography on the Carbowax column. The areas of the peaks, their retention time, and elution temperature were recorded.

K. Large Scale Separation of the Organic Acids of Bryophyllum calycinum

Five grams of Bryophyllum calycinum stock tissue were placed in each of four 250 ml stainless steel centrifuge bottles. To each bottle, 45 ml of thoroughly washed Bio-Rex 50 W H^+ resin (20 - 50 mesh), 150 ml of deionized water, and a magnetic stirring bar were added. The tissue was extracted with rapid stirring for 19 hours at 40 - 45°C.

The bottles were centrifuged at 7,000 R.P.M. for 10 minutes, and the supernatants were combined and passed through Whatman #1 filter paper on a Buchner funnel. Water (150 ml) was added to the residue in each bottle and the mixture was stirred rapidly for 15 minutes. Centrifugation of the solution at 8,000 R.P.M. for 5 minutes and filtration of the supernatant through filter paper was performed. Extraction of the tissue, centrifugation of the solution, and filtration of the supernatant were carried out two more times. The total filtered supernatant solution was filtered through a 0.8μ pore Millipore filter.

A 570 ml Bio-Rad AG 1 x 8 (20 - 50 mesh) resin column was prepared in the carbonate form by conversion from the hydroxide form. The ratio of length to width of the column was 10 : 1. The total filtered extract was passed slowly through this column and the column was thoroughly rinsed with water. The acids were eluted by approximately linear gradient elution with an increase in concentration of $(NH_4)_2CO_3$ from 0 to 1.7 N. The apparatus for generating the gradient consisted of two large containers connected horizontally at the bottom. The container of eluant

was stirred with a magnetic stirrer. Two hundred, 125 ml fractions were collected at a flow rate of one bed volume every 23 minutes (25 ml/min). The fractions were stored at 4°C.

A 1 ml aliquot of every third fraction was dried at 72-73°C. These samples, dissolved in water, were spotted on two sets of silica gel plates. One set of plates was developed in t-amyl alcohol : acetic acid, 25 : 10. The second set was developed in 95% ethanol : NH_3 : H_2O, 20 : 3 : 2. The plates were sprayed with basic $KMnO_4$ spray reagent. The spots were marked and R_f values measured.

The fractions were pooled according to the acids apparently present in each fraction as determined from the thin-layer results, and each pooled fraction was dried in vacuo at 61 - 64°C on a rotary evaporator. Small amounts of water were added in succession to each dried fraction to dissolve the acids and successive 5 ml solutions were prepared from each fraction so that the concentration of acids would be high in the first solution and decrease through succeeding solutions. Samples of each of these solutions, the amount depending on the amount of acid assumed to be present, were titrated with standard NaOH using thymol blue - phenolphthalein indicator. These solutions were then titrated with 0.02 N HCl to the end point of thymol blue in its acid range (pH 2.75). The milliequivalents of acid in each solution were calculated.

All of those solutions which contained greater than 40% of the total acid of each fraction were spotted in their entirety 2 cm from the bottom, on 11¼" x 8-3/4" sheets of Whatman #17 preparative chromatography paper. Fifty spots of 1 µl placed 4 mm apart formed the main band of acid and five spots separate on the right formed a band of acid for

detection purposes. The 1 μl applications were repeated until all the solution had been applied. The number of sheets used for each fraction depended on the amount of acid in each fraction. The sheets were developed in ether : formic acid : H_2O, 5 : 2 : 1, in jars saturated with solvent vapour.

The right hand region of the chromatograms was cut off and sprayed with 0.04% bromphenol blue made just basic. The spots were marked and R_F values were recorded.

The chromatograms were cut into horizontal sections according to these results. The acids in these strips were eluted by downward descent of 3N NH_4OH. The eluates were dried at 60 - 75°C. These fractions or a portion of these fractions were transferred to culture tubes using deionized water and the solutions in the tubes were dried.

The apparent amount of acid in each fraction, estimated from the chromatogram results, determined the amount of resin added to each tube. Methanol was added to the tubes containing resin to completely wet the resin and provide an excess for gas chromatography. The acids were esterified using the autoclave. Each esterified fraction was examined by gas chromatography on the 600 B chromatograph equipped with the Carbowax column, at 100°C, 150°C, and 210°C.

The supernatant of each fraction was injected into the A-100 gas chromatographic instrument equipped with the SE-30 column, and the esters were separated by programmed temperature oven heating. The detected substances were collected, and were examined by infrared spectroscopy.

Organic acid standards were esterified, and the esters were passed through the A-100 gas chromatograph, collected, and examined by infrared spectroscopy.

CHAPTER III

RESULTS AND DISCUSSION

A. Extraction of Plant Material

Using the method outlined previously, 0.1 g samples of Bryophyllum calycinum dried leaf tissue were extracted with water in the presence of H^+ form resin. The final volume of each extract was 10 ml. Two ml aliquots of these extracts were titrated with 0.04014 N NaOH and the μeq of acid present in the dried leaf tissue were calculated. The results are presented in Table II. Extractions A and B were carried out at the same time; extraction C was carried out on a separate occasion to provide an extract for thin-layer chromatography.

The agreement of the values for μeq/g of tissue both between samples in one extraction and between separate extractions indicates that the method of extraction gives reproducible results. These results indicate that 16 hr of extraction are sufficient to remove the organic acids from the tissue. The extraction probably removes all the acid in the leaf tissue, although it would be necessary to add a known amount of acid to the tissue before extraction and then determine the recovery of this acid in order to elucidate the efficiency of the extraction method. However, the number of μeq of acid extracted is in excellent

TABLE II

AMOUNT OF ORGANIC ACIDS EXTRACTED FROM BRYOPHYLLUM CALYCINUM LEAF TISSUE

Extraction	Period of Extraction, hr	Weight of Tissue, g	Titration, ml 0.04014 N NaOH	Amount of Acid, μeq/g dry weight of leaf tissue
	16	0.0000	0.02	0
			0.02	
A	16	0.0993	1.34	2668
			1.34	
	16	0.1008	1.37	2693
			1.375	
	39	0.0000	0.0325	0
			-	
B	39	0.0998	1.36	2677
			1.3675	
	39	0.0999	1.36	2667
			1.36	
	16	0.0000	0.025	0
			0.02	
C	16	0.1001	1.35	2657
			1.345	
	16	0.1001	1.31	2581
			1.31	

agreement with the value obtained by Vickery and Wilson (102).

B. Esterification

Known organic acids were esterified in the autoclave after methanol and H^+ form resin (156 mg resin / 100 μeq acid) had been added to each acid sample in an 8 ml culture tube and the tubes had been closed.

In esterification A, the 2 ml aliquots of acid stock solutions taken for esterification were dried in the culture tubes at 50°C under an air stream before the addition of resin and methanol. After esterification, the ester solution was removed from the resin by filtration and made to a volume of 5 ml. One ml aliquots were dried and titrated directly to determine the free acid remaining after esterification. After 1 ml aliquots had been saponified, passed through Dowex 50 H^+ and dried, they were titrated to determine the acid recovered after esterification. One ml aliquots of the acid stock solutions were dried at 50°C in an air stream and titrated to determine the acid present in the tubes prior to esterification. The results of these titrations, and the percentage esterifications and recoveries of the various acids are presented in Table III.

In esterification B, 1 ml aliquots of methanolic acid stock solutions were esterified directly after the addition of resin and a small magnetic stirring bar. The solutions were stirred during esterification to ensure mixing of the acid solution, although this may not have been necessary, since Bochner et al. (8) found that the rate of bulk diffusion of acid from the external solution to the resin catalyst was not the rate-limiting step in esterification. Each solution of

ester was removed from the resin by filtration and a 0.1 ml aliquot of this solution was titrated. The ester in this 0.1 ml aliquot was saponified and the resulting solution was passed through Dowex 50 H^+ and titrated. A 1 ml aliquot of each of the acid stock solutions was titrated to determine the acid available for esterification. The results of this esterification are presented in Table III.

The esterification of the acids was essentially complete with the method used. The percentage esterification values for esterification B were generally lower than those of esterification A. A smaller proportion of the acid esterified was used in the former for titration of the free acid remaining after esterification than in the latter. Any slight over-titration in esterification B is thus magnified in the calculation of percentage esterification.

The apparent percent esterifications of α-ketoglutaric acid and pyruvic acid are lower than those of other acids, except for oxalic. When the ester solutions of these acids are titrated it is difficult to obtain an accurate end-point since the colour of the indicator changes very rapidly from the pale purple of the end-point to yellow. This is presumably the result of production of acid by hydrolysis of the ester. If saponification of these esters during titration could be prevented, the percentage esterification would probably be found to be higher than the results indicate. The low percent esterification of oxalic acid is probably related to the low pKa values of this acid. Oxalacetic acid is a relatively unstable compound and it may be that reactions other than esterification occur during the heating in the autoclave. This might lead to the esterification of only 80% obtained for this acid.

TABLE III

ESTERIFICATION AND RECOVERY OF ORGANIC ACIDS

Acid	Esterification A					Esterification B				
	Titration*			Percent	Percent	Titration			Percent	Percent
	Initial[a]	Ester	Final	Ester	Recovery	Initial[b]	Ester	Final	Ester	Recovery
cis-Aconitic	0.640	0.015	0.725	98.3	113.0	0.305	0.02	0.2775	93.4	91.0
	0.648	0.01	0.730			0.305				
Citric	0.764	0.01	0.735	98.6	96.7	0.3015	0.02	0.2875	93.4	95.1
	0.756	0.01	0.735			0.303				
Fumaric	0.758	0.00	0.735	100.0	100.3	0.3145	0.0275	0.2975	91.2	94.7
	0.758	0.00	0.745			0.314				
Glutaric	0.716	0.00	0.715	99.7	100.3					
	0.714	0.005	0.720							
Glycolic						0.263	0.0125	0.1975	95.2	75.4
						0.261				
Glyoxylic						0.1475	0.0175	0.1375	88.1	93.4
						0.147				

TABLE III (Continued)

Acid	Esterification A					Esterification B				
	Titration*			Percent	Percent	Titration			Percent	Percent
	Initial[a]	Ester	Final	Ester	Recovery	Initial[b]	Ester	Final	Ester	Recovery
Isocitric						0.191	0.005	0.1275	97.4	66.8
						0.19075				
Itaconic	0.756	0.005	0.735	99.7	97.6					
	0.756	0.00	0.740							
α-Ketoglutaric	0.604	0.08	0.605	86.1	100.2	0.265	0.07	0.2075	73.7	77.9
	0.614	0.09	0.615			0.268				
Lactic						0.226	0.01	0.1775	95.6	78.5
						0.226				
Malic	0.398	0.025	0.540	95.8	133.0	0.2905	0.01	0.2775	96.5	96.4
	0.414	0.02	0.540			0.2855				
Malonic	0.608	0.005	0.575	99.6	94.3	0.2875	0.015	0.2775	94.8	96.9
	0.612	0.00	0.575			0.2855				
Oxalacetic						0.113	0.0225	0.0875	80.1	77.4
						0.113				

TABLE III (Continued)

Acid	Esterification A					Esterification B				
	Titration[a]			Percent	Percent	Titration			Percent	Percent
	Initial	Ester	Final	Ester	Recovery	Initial[b]	Ester	Final	Ester	Recovery
Oxalic						0.31275	0.115	0.1750	63.3	55.8
						0.31425				
o-Phthalic	0.790	0.02	0.800	97.5	101.1					
	0.788	0.02	0.795							
Pyruvic	0.576	0.095	0.505	81.7	87.4	0.281	0.075	0.3125	73.3	111.2
	0.580	0.09								
Succinic	0.748	0.00	0.735	100.0	98.6	0.278	0.01	0.2625	96.4	94.3
	0.748	0.00	0.740			0.27875				
Tartaric	0.724	0.04	0.725	93.8	99.3	0.253	0.0175	0.28	93.0	111.3
	0.726	0.05	0.715			0.250				
Tricarballylic	0.790	0.05	0.835	95.8	104.9					
	0.790	0.02	0.825							

TABLE III (Continued)

[a] The titration values for the original acid solution have been multiplied by 0.4 to correspond to the values obtained for the ester solution.

[b] The titration values for the original acid solution have been multiplied by 0.1 to correspond to the values obtained for the ester solution.

* All titrations are ml of 0.04014 N NaOH.

Since single acids are effectively esterified with the method used, mixtures of acids should also show essentially complete esterification.

The values for percent recovery indicate that the acids may be satisfactorily recovered from the ester solution. The lower values obtained in esterification B may be a result of the assumption, for the purposes of calculation, that the ester solution present after the esterification of 1 ml of acid solution was exactly 1 ml. If this was not so, variation in the final volumes may explain the lower percent recoveries obtained and the wide variation in these values.

The reason for the high recovery values for cis-aconitic and malic acids in esterification A is not known. Oxalic acid shows a low recovery value in esterification B and this may be related to the low percentage esterification. Although the dimethyl ester may be recovered from the resin, the half-ester and free acid may remain associated with the H^+ groups of the resin and this will lead to a low percentage recovery.

C. Analytical Gas Chromatography of Known Esters

Gas chromatographic analysis of the esters prepared from known acids using the resin technique was carried out on the Aerograph 600B gas chromatograph equipped with the Carbowax 20 M column. The relationships between the retention volumes of the esters and the column temperatures are presented in Figure 1. The retention volume (ml) at a particular column temperature was the retention time (min) of the ester multiplied by the flow rate of the nitrogen carrier gas (ml/min). The retention time was measured from the apparent leading edge of the

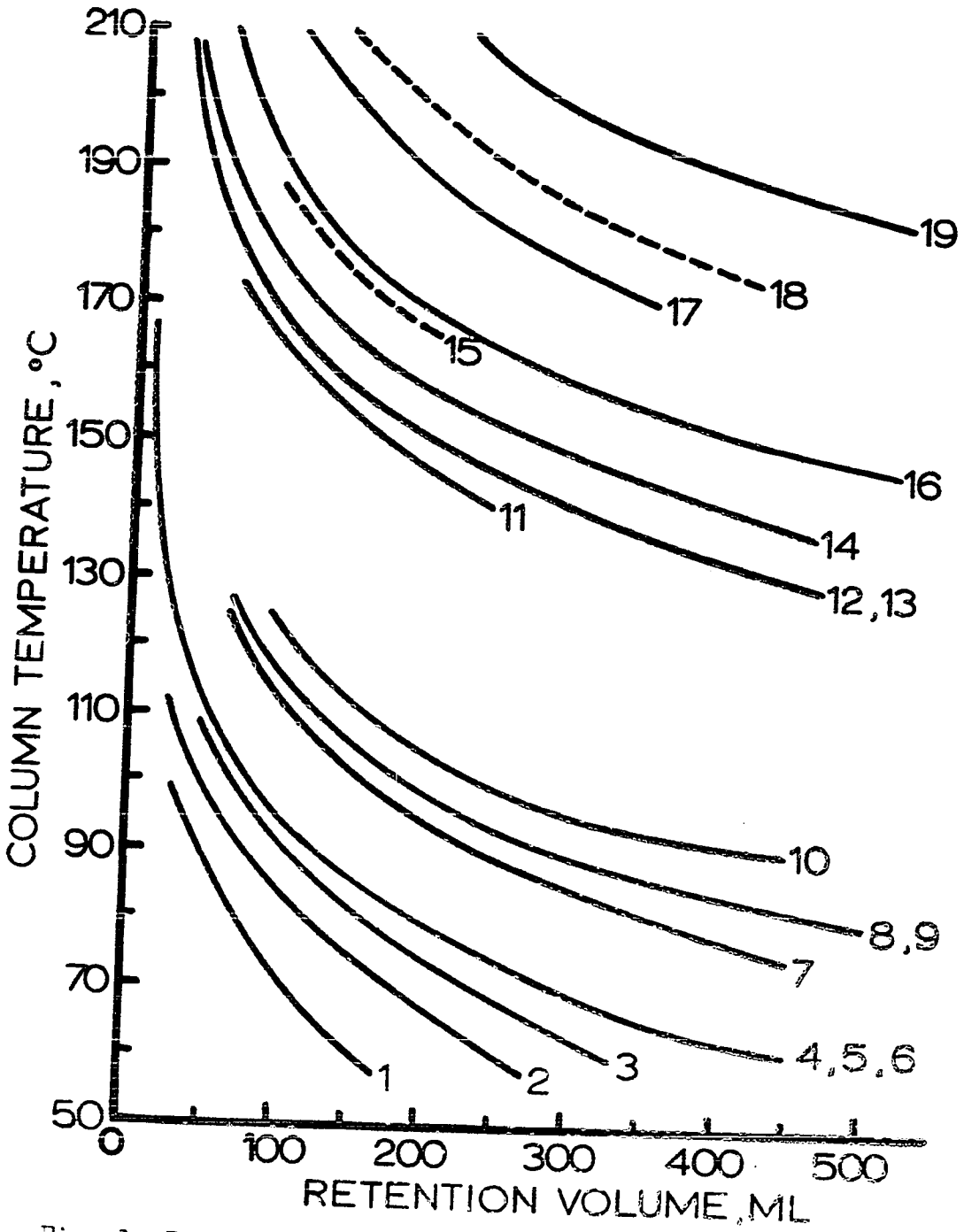


Fig. 1. Retention volumes on Carbowax 20 M of organic acid methyl esters. Methyl esters of: (1) oxalacetic (peak 1), (2) lactic, (3) glycolic, (4) glyoxylic, (5) oxalic, (6) oxalacetic (peak 2), (7) malonic, (8) pyruvic, (9) fumaric, (10) succinic, (11) oxalacetic (peak 3), (12) α-ketoglutaric (peak 1), (13) malic (peak 1), (14) α-ketoglutaric (peak 2), (15) tartaric, (16) cis-aconitic, (17) citric, (18) malic (peak 2), (19) isocitric.

methanol solvent peak; this leading edge represents the air peak. Measurement from the air peak rather than the time of injection eliminates the need to correct for the "dead volume" of the column in determining the retention volume. Flow rate was 40.8 ml/min at 20°C.

The esters of known acids prepared by the method of esterification described previously were analyzed on the A-100 gas chromatograph equipped with the silicone column in a later experiment where the esters were collected for infrared analysis. The order of elution of these esters from the silicone column and their elution temperatures are presented in Table IV. Table XXII, p.92, gives operating conditions.

The order of elution with both Carbowax and silicone columns, in general, follows the sequence of the boiling points of the esters, as would be expected. Other work has indicated that the order of elution of the esters follows that of their boiling points on both polar and non-polar columns.

The elution of methyl pyruvate would be expected in the vicinity of methyl lactate, according to the boiling point of methyl pyruvate. However, methyl pyruvate was not eluted until considerably later. Alcock (2) also found that methyl pyruvate was eluted after methyl lactate on a diethylene glycol column. Simmonds et al. (87) obtained a derivative of methyl pyruvate with a higher boiling point, methyl 2, 2 - dimethoxypyruvate, when pyruvic acid was esterified in methanol containing HCl, but the compound formed in the present work apparently has an even higher boiling point, possibly 200 - 210°C. Methyl pyruvate may be formed in the course of the resin-catalyzed esterification but at the high reaction temperature used, this compound may undergo further reaction to give the final product, whose boiling point suggests

TABLE IV

ELUTION OF THE METHYL ESTERS OF ORGANIC ACIDS FROM A SILICONE COLUMN

Methyl Ester of the Acid	Elution Temperature, °C	Boiling Point of the Ester, °C
Oxalacetic (peak 1)	70	
Glyoxylic	86	
Glycolic	93	151.2
Lactic	100	144.8
Oxalacetic (peak 2)	109	
Oxalic	111	163.3
Malonic	126	181
Fumaric	147	192
Succinic	147	192.8
Pyruvic	153	137
Oxalacetic (peak 3)	158	
Malic	162	242
α-Ketoglutaric (peak 1)	169	
Tartaric	171	280
α-Ketoglutaric (peak 2)	189	
cis-Aconitic	193	270
Citric	198	287
Isocitric lactone	202	

that it is not a simple derivative of pyruvic acid.

Three compounds are evident when the ester solution resulting from esterification of oxalacetic acid is subjected to gas chromatography on a Carbowax or silicone column. The first and third compounds to be eluted occur in approximately equal proportions and each is present in an amount approximately one-tenth that of the second compound to be eluted, the major product of the reaction.

Gas chromatography of the compounds produced from oxalacetic acid during esterification has previously yielded a variety of results. Kuksis and Vishwakarma (50) obtained three compounds on a Carbowax 20 M column when oxalacetic acid was esterified with either diazomethane or methanol containing boron trifluoride. The compounds in the order of their elution, were considered to be the keto form of dimethyl oxalacetate, the enol form of dimethyl oxalacetate, and the methyl ether of the enol ester of dimethyl oxalacetate. In later work, using similar methods of esterification and a Carbowax column, Kuksis and Prioreschi (49) obtained a single peak for dimethyl oxalacetate. Single compounds were also obtained from oxalacetic acid after esterification by Kowala et al. (47) and Kellog et al. (41). However, these compounds differed in their elution from a silicone column. Simmonds et al. (87) obtained three compounds from oxalacetic acid when it was methylated with methanol-HCl reagent and these were, in the order of their elution; dimethyl oxalacetate, dimethyl 2, 2 - dimethoxysuccinate, and an unidentified trace component. The amount of the second compound increased as the esterification temperature was raised.

Two of the three compounds produced from oxalacetic acid in the present work were eluted from both the Carbowax and silicone columns before

the esters of malonic, fumaric, and succinic acids. This early elution suggests that these compounds are the result of breakdown of oxalacetic acid during esterification. The first compound eluted is thought to be methyl pyruvate since the retention volume of the compound on both the Carbowax and silicone columns is similar to that which would be expected for methyl pyruvate, considering the boiling point of methyl pyruvate and its structure. This compound could be produced as a result of the decarboxylation of oxalacetic acid which can occur spontaneously in solution (52, p.227) and which may be accelerated by the high temperature of the esterification method. Graham and O'Reilly (32) have reported extensive decarboxylation of oxalacetic acid when this compound was analyzed through gas chromatography of its methyl ester.

The second compound and major product of the reaction is possibly methyl 2, 2 - dimethoxypyruvate. The reaction of pyruvic acid, produced by decarboxylation of oxalacetic acid, to produce this compound would be expected by analogy with the acid-catalyzed esterification of pyruvic acid carried out by Simmonds et al. (87). The retention volume of methyl 2, 2 - dimethoxypyruvate on a Carbowax or silicone column should agree with that of the unknown compound.

The third compound is apparently a derivative of the enol form of dimethyl oxalacetate since the infrared spectrum indicates the presence of a hydroxyl group but shows no double bond absorption. The compound may be the methoxy derivative : dimethyl 2 - hydroxy - 2 - methoxysuccinate. This compound should be eluted from Carbowax and silicone columns in a similar manner to the third compound produced during esterification of oxalacetic acid.

α-Ketoglutaric acid gave two compounds when the ester solution was analyzed by gas chromatography. Alcock (2) and Graham and O'Reilly (32) obtained two compounds when this acid was esterified. Simmonds et al. (87) found that the reaction of α-ketoglutaric acid with methanol - HCl yielded two products in approximately equal amounts. The first compound eluted in gas chromatography was dimethyl α-ketoglutarate and the second, dimethyl 2, 2 - dimethoxyglutarate.

The first compound eluted from the gas chromatography columns in the present work is apparently dimethyl α-ketoglutarate.

The second compound obtained on the Carbowax column may not be the same as that obtained on the silicone column since the elution of these compounds differed markedly. Two separately prepared ester solutions were used so this may be the basis of the difference. As well, the proportions of the first and second compounds differed markedly between preparations; in the ester solution analyzed on the Carbowax column the amount of the first compound was very small, whereas in the ester solution analyzed on the silicone column the compounds were present in approximately equal amounts. The second compound found on the Carbowax column may be dimethyl 2, 2 - dimethoxyglutarate which will have a higher boiling point than dimethyl α - ketoglutarate and should have a retention volume similar to the unknown compound. The second compound eluted from the silicone column is apparently a compound with a higher boiling point than dimethyl 2, 2 - dimethoxyglutarate.

The ester of isocitric acid obtained after esterification with ion exchange resin in methanol is the dimethyl ester of isocitric lactone. When isocitric lactone is esterified, a single peak is obtained with the same retention time on a silicone column as that for

esterified isocitric acid. As well, the infrared spectrum of the methyl ester of isocitric acid indicates the presence of the lactone ring and matches the spectrum for dimethyl isocitric lactone. It is apparent that lactonization occurred during esterification and this has also been observed by Kellog et al. (41) who found that ester preparations of both isocitric acid and isocitric lactone gave a single ester with a retention time greater than trimethyl citrate and identical to that of authentic dimethyl isocitric lactone. Kuksis and Prioreschi (49) observed that the methyl ester of isocitric lactone was eluted after trimethyl citrate on Carbowax 20 M. With diazomethane as the esterification reagent, Canvin (16) obtained trimethyl isocitrate which was eluted between trimethyl citrate and dimethyl isocitric lactone.

Two compounds are eluted from a Carbowax 20 M column when an ester solution of malic acid is separated but only one compound is found using a silicone column. The compound eluted first from the Carbowax column is apparently dimethyl malate. The structure of the second compound eluted is not known. The position of the second malic peak is not marked definitely in Figure 1 since its retention volume appears variable. When constant temperatures are used, the retention volumes obtained for this compound seem constant. However, when temperature programming is used starting from 60°C, the second compound for malic acid is not eluted until later than would be expected and comes off the column at the same time as dimethyl isocitric lactone. This variability in retention volume and the fact that no second compound occurs when a silicone column is used, indicate that an interaction probably occurs between dimethyl malate and Carbowax 20 M which results in the production of the unknown compound.

Tartaric acid after esterification gives a peak of the expected size on a silicone column but gives an extremely small peak on the Carbowax column. Again, it appears an interaction occurs between dimethyl tartrate and Carbowax 20 M. Since both dimethyl malate and dimethyl tartrate show this interaction, it seems the reaction may be related to the presence of hydroxyl groups in the molecules.

D. Separation of the Organic Acids of Bryophyllum calycinum on Silica Gel Thin Layer Plates.

Two aliquots of 1 ml were taken from 10 ml of extract prepared from 0.1 g of tissue. The aliquots were titrated with NaOH solution, dried, taken up in water, and spotted on silica gel plates as described previously. The plates were developed in the acidic solvent t-amyl alcohol : acetic acid, 25 : 10; and, after drying, portions of the plates were sprayed with basic potassium permanganate reagent. The results of silica gel thin-layer chromatography of the samples are shown in Figure 2. The R_f values of the acids and a description of the appearance of the acid spots indicated in the figure are given in Table V.

The separation of acids was similar on the chromatograms of both aliquots but the R_f values from the second set of chromatograms were higher than those from the first set. Since the two sets of plates were prepared separately from two preparations of silica gel slurry, the differences in R_f values probably reflect differences in the substrate layers.

TABLE V

SILICA GEL THIN-LAYER CHROMATOGRAPHY OF THE ORGANIC ACIDS OF BRYOPHYLLUM CALYCINUM

Spot	Range of R_F Values (one plate)*	Description of Spot	Probable Acid
a	4.0 - 4.5	very pale yellow-pale yellow	
	5.4 - 5.9		
b	8.3 - 9.6	pale yellow	
	9.6 - 9.7		
c	11.5 - 13.1	bright yellow	
	13.8 - 14.6		
d	14.9 - 17.2	yellow-medium yellow	
	18.1 - 18.3		
e	19.2 - 21.7	medium yellow	
	23.7 - 24.8		
f	22.8 - 26.3	very pale yellow	
	28.7 - 32.2		
g	65.6 - 73.5	bright yellow (tails back)	Malic+Citric
	69.3 - 71.9		+Isocitric
h	97.9 - 99.2	yellow	Succinic
	99.5 -100.0		

* The two sets of values represent values from two sets of plates.

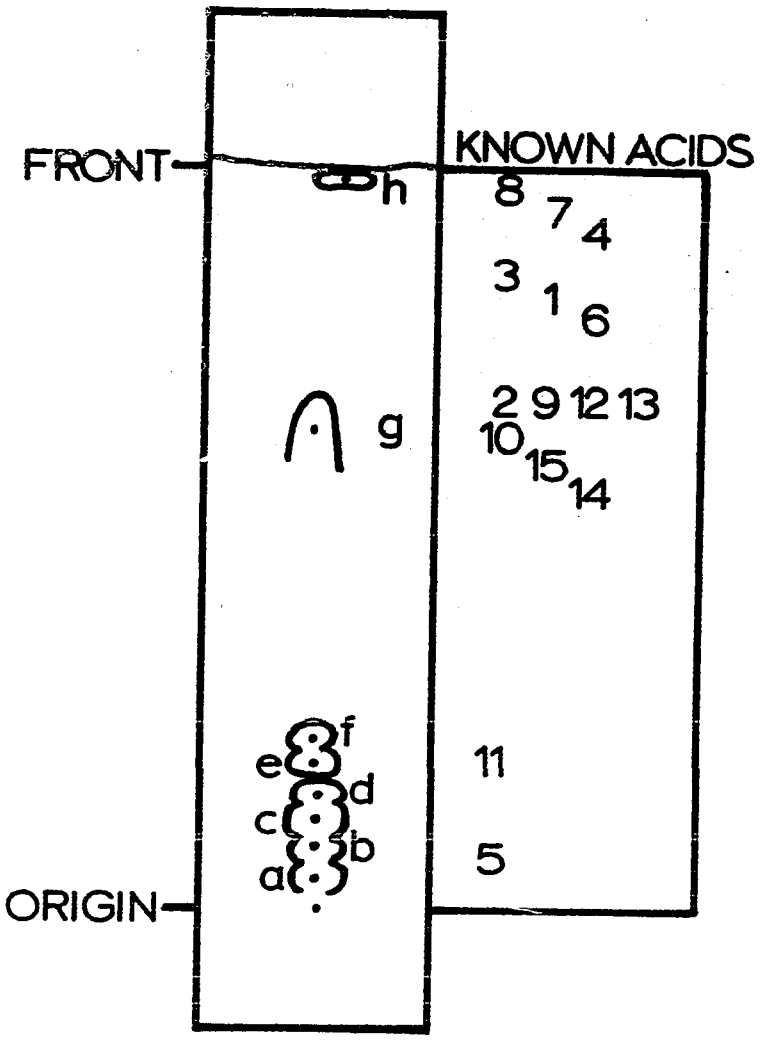


Fig. 2. Silica gel thin-layer separation of the organic acids of Bryophyllum calycinum. Approximate R_F of known acids: (1) glyoxylic, (2) glycolic, (3) lactic, (4) pyruvic, (5) oxalic, (6) malonic, (7) succinic, (8) fumaric, (9) oxalacetic, (10) malic, (11) tartaric, (12) α-ketoglutaric, (13) cis-aconitic, (14) citric, (15) isocitric.

Attempts which were made to elute the organic acids from the silica gel taken from unsprayed plates were not successful. Regions of the silica gel from one set of plates were extracted with methanol, which removed those acids having a high R_f value but failed to elute malic acid. The use of 3N NH_4OH for the elution of acids from the silica gel of the second set of plates resulted in the extraction of large amounts of acidic material which interfered with determination of the amounts of acid and prevented satisfactory esterification of the eluted acids. Preliminary separation of organic acids on silica gel thus does not appear satisfactory if the separated acids are to be studied further. Relatively non-polar eluants fail to remove tightly held acids whereas polar eluants remove undesired acidic material.

E. Separation of the Organic Acids of Bryophyllum calycinum by Cellulose Thin-Layer Chromatography and Gas Chromatography.

Since silica gel thin-layer chromatography presented difficulties as a method for the preliminary separation of the organic acids in the plant extract, the organic acid extract was separated initially on cellulose thin-layer plates.

A 2 ml aliquot was removed from a 10 ml extract prepared from 0.1 g of tissue. This aliquot was neutralized with 0.04 N NaOH, dried, taken up in a minimum amount of water, and spotted on cellulose plates as previously described. The plates were developed in acidic and basic solvents; one-half the total number was placed in each solvent. After the cellulose had dried, portions of the plates were sprayed with acidic potassium permanganate reagent to detect the acids. The

results of the separation of the organic acids from the plant extract on microcrystalline cellulose plates with basic and acidic solvents are presented in Figure 3. The R_F values of the acids and a description of the spots appearing on the chromatograms are presented in Table VI.

TABLE VI

CELLULOSE THIN-LAYER CHROMATOGRAPHY OF THE ORGANIC ACIDS OF BRYOPHYLLUM CALYCINUM

Solvent	Spot	R_F Value Range *	Description of Spot	Probable Acid
Acidic	a	10.2-12.7	very pale white	
	b	13.1-16.8	very pale white	
	c	18.0-21.8	brown,darker than background	Sodium ion
	d	45.3-48.9	strong white	Citric+Isocitric
	e	49.4-53.4	strong white	Malic
Basic	a	3.5- 5.0	strong white	Citric+Isocitric
	b	6.2- 7.2	strong white	Citric+Isocitric
	c	13.2-15.4	very pale white	
	d	17.7-20.8	medium strong white	Malic
	e	19.9-23.8	medium strong white	Malic
	f	61.1-64.9	very pale white	

* R_F value range represents the maximum difference between plates.

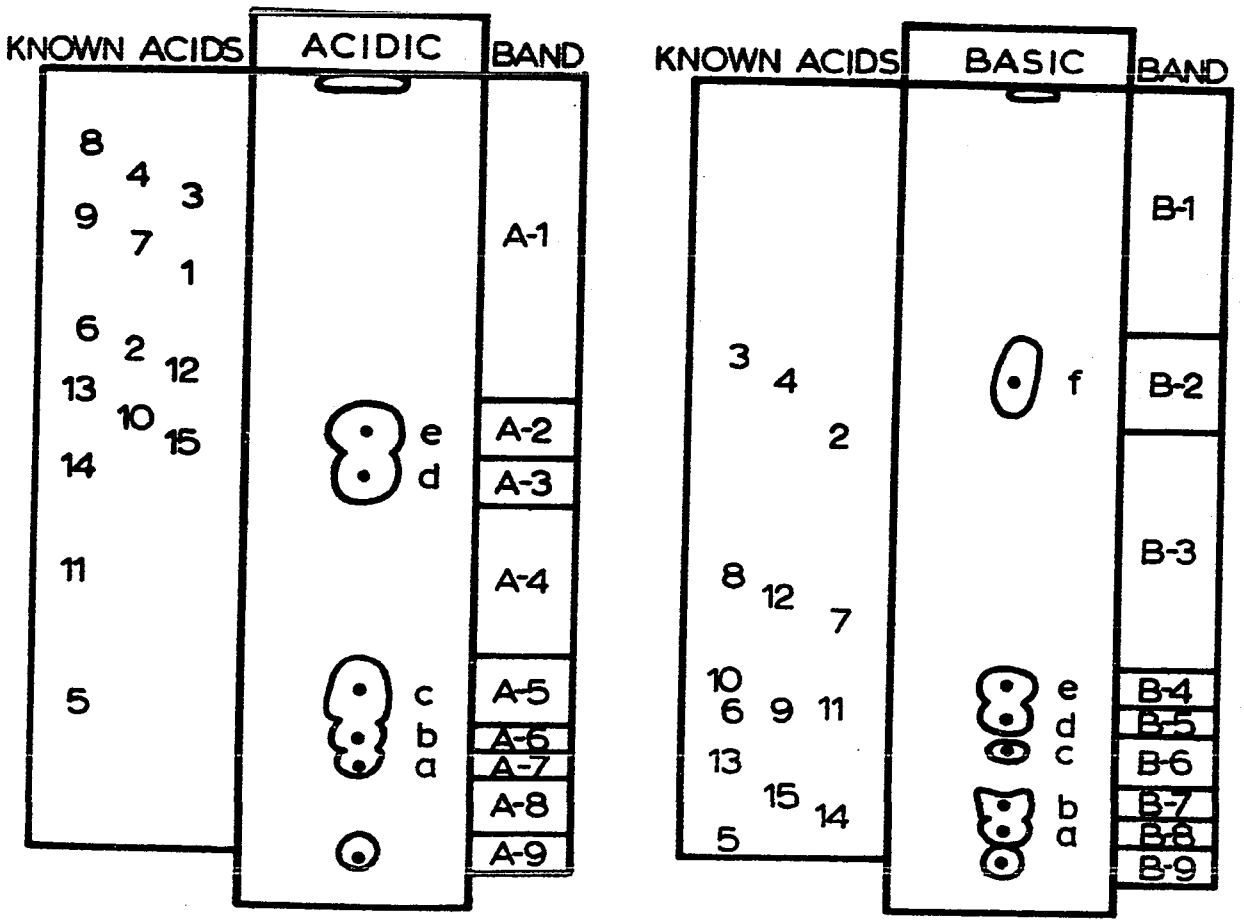


Fig. 3. Cellulose thin-layer separation of the organic acids of Bryophyllum calycinum. Approximate R_F of known acids: (1) glyoxylic, (2) glycolic, (3) lactic, (4) pyruvic, (5) oxalic, (6) malonic, (7) succinic, (8) fumaric, (9) oxalacetic, (10) malic, (11) tartaric, (12) α-ketoglutaric, (13) cis-aconitic, (14) citric, (15) isocitric.

After the position of the acids on the cellulose substrate had been determined, the unsprayed regions of cellulose were divided into horizontal bands, to allow the recovery of the acid in each particular band. The positions at which the divisions were made are indicated in Figure 3. The acids in corresponding bands were extracted with 3N NH_4OH and the extracts were finally dried in culture tubes in preparation for esterification.

The percentage of the total acid present in each fraction was estimated from the appearance of the spots on the chromatograms and the amount of acid which should be present in each fraction was then calculated from the total amount of acid available for elution, based on titration values for the original plant extract. The estimates of the amount of acid in each fraction and the amounts of resin and methanol added for the esterification of the acids are presented in Table VII. The amount of resin added was greater than that previously used, in proportion to the amount of acid assumed present, since the percentage of acid in some fractions may have been underestimated and since the presence of NH_4^+ ion in all fractions and Na^+ ion in some fractions would reduce the catalytic activity of the resin.

After the acids had been esterified, a small aliquot of each ester solution was analyzed by gas chromatography on the Carbowax 20 M column. The conditions used in the gas chromatographic analysis of the esterified fractions are presented in Table VIII.

TABLE VII

ESTIMATED AMOUNTS OF ORGANIC ACIDS AVAILABLE FOR ESTERIFICATION

Fraction	Spot	Estimated Percent of Total Acid	Estimated Amount of Acid, μeq	Amount of Resin Added, mg	Amount of Methanol Added, μl
A-1		3.5	0.54	-	-
A-2	e	35.1	5.43	-	-
A-3	d	35.1	5.43	25.1	200
A-4		3.5	0.54	5.2	60
A-5	c	3.5	0.54	6.5	70
A-6	b	5.3	0.82	6.7	60
A-7	a	5.3	0.82	4.9	60
A-8		3.5	0.54	2.9	40
A-9		5.3	0.82	4.2	50
B-1		3.9	0.71	3.1	60
B-2	f	5.9	1.07	4.3	50
B-3		3.9	0.71	2.5	40
B-4	e	13.7	2.48	10.9	80
B-5	d	13.7	2.48	11.5	80
B-6	c	5.9	1.07	8.4	60
B-7	b	23.5	4.25	17.2	90
B-8	a	23.5	4.25	15.5	90
B-9		5.9	1.07	4.5	50

TABLE VIII

CONDITIONS FOR SEPARATION OF ORGANIC ACID METHYL ESTERS ON A CARBOWAX 20 M COLUMN

Sample amount	1.5 - 2.5 μl
Initial temperature of oven	60 - 80°C
Final temperature of oven	220°C
Time for temperature increase	60 min
Oven power for temperature increase	75
Time oven power set at 75	5 min after injection
Injection block temperature	200°C
Hydrogen flow	25 ml/min
Nitrogen flow	38.5 ml/min
Temperature for nitrogen measurement	21.5°C
Input impedance	10^9
Output sensitivity	1 X
Attenuation	1 X
Range of recorder	1 mv
Chart speed	15"/hr

The elution temperatures of the methyl esters found in the esterified fractions and the identity of the esters are presented in Table IX.

TABLE IX

GAS CHROMATOGRAPHY OF METHYL ESTERS OF ORGANIC ACIDS FROM BRYOPHYLLUM CALYCINUM

Fraction	Elution Temperature, °C	Probable Ester
A-3	184	Trimethyl citrate
A-3	218	Dimethyl isocitric lactone
B-5	162	Dimethyl malate
B-7	185	Trimethyl citrate
B-7	220	Dimethyl isocitric lactone
B-8	217.5	Dimethyl isocitric lactone
B-9	218	Dimethyl isocitric lactone

Only the esters of major acids were detected since the acids separated as their methyl esters by gas chromatography represented the acids of 6 to 7 mg of dried leaf tissue, approximately 17 μeq of acid. The amounts of these esters indicated that extraction of the acids from cellulose with 3N NH_4OH was satisfactory.

Unidentified peaks occurred in all fractions and in some cases were larger than the ester peaks. Unknown compounds which were present in all fractions evidently originated from the cellulose extracted after thin-layer chromatography. Other compounds, present only in certain fractions, may represent products of the breakdown during esterification of sugars or other material in the unpurified extract.

F. Separation of the Organic Acids of Bryophyllum calycinum.

Since artifact compounds may apparently arise from impurities in the cellulose or the breakdown during esterification of sugars or other material in the extract, steps were taken to avoid these possibilities. The cellulose was extracted with 5N NH_4OH to remove impurities before the plates were coated. The plant extract was passed onto a carbonate form anion exchange column which adsorbed the organic acids. The sugars and other neutral material in the extract were washed from the column with water and the organic acids were eluted with $(NH_4)_2CO_3$ solution, yielding a purified organic acid extract.

The plant extract of 500 ml was prepared from 5 g of dried Bryophyllum leaf tissue as described previously. One hundred ml of this extract were purified on the carbonate form anion exchange column and the organic acid eluate was dried to remove the $(NH_4)_2CO_3$. The ammonium salts of the organic acids were dissolved in water and the solution was made to a volume of 10 ml.

Four ml aliquots of the original extract were titrated with 0.04014 N NaOH and 0.4 ml aliquots of the purified extract were titrated after they had been passed through columns of Dowex 50 H^+. The titration results and the percentage recovery of the organic acids from the anion exchange column are shown in Table X.

TABLE X

RECOVERY OF THE ORGANIC ACIDS OF *BRYOPHYLLUM CALYCINUM* FROM CARBONATE FORM ANION EXCHANGE RESIN

Titration of Original Extract	Grams of Tissue Extracted	Amount of Acid, μeq/g tissue	Titration of Purified Extract	Percentage Recovery of Acid
2.76925	5.0406	2754	2.675	97.2
2.76425			2.705	

The results indicate that the expected amount of acid was extracted from the 5 g of leaf tissue. The recovery of acids after passage through the carbonate form anion exchange column was very good and apparently acids were not retained on the column. The resin column will remove phenolic acids present in the extract and the loss of these may cause the slight decrease in the recovery value. Kinzel (43) found that since phenolic acids are auto-oxidizable in the alkaline range, they oxidise on the column. The products of oxidation remain on the column. As well, phosphorylated acids will probably also be retained on the column since Aronoff (3, p. 119) doubted whether even sodium carbonate would be sufficiently alkaline to remove these acids. The efficient recovery of acids agrees with the results of Resnik *et al.* (78) who found that with 1.5 N $(NH_4)_2CO_3$ as eluant, the recovery of all acids from carbonate form anion exchange resin was excellent. The recovery of succinic and fumaric acids was 99%; of malic and citric acids, 97%; and of oxalic acid, 96%.

A total of 2.0 ml of the purified extract was spotted as bands on microcrystalline cellulose plates. These plates were developed in acidic or basic solvent and after the cellulose had dried, the location of the acids was determined by spraying a region of the plates with acidic potassium permanganate solution. The results of thin-layer chromatography of the purified organic acid extract on preparative microcrystalline cellulose plates are presented in Table XI and Figure 4.

The separation on these preparative plates was not as distinct as that obtained previously on cellulose, possibly because these plates were loaded too heavily with acid.

The unsprayed portions of cellulose were divided into horizontal bands as indicated in Figure 4 in order to isolate the acid fractions and enable further separation of the acids in each band. The corresponding bands from the cellulose plates were combined and the acids were extracted from the cellulose with 3N NH_4OH. After the fractions prepared from corresponding bands of cellulose had been dried, passed through Dowex 50 H^+, and again dried, they were dissolved in 30 μl of methanol.

Silica gel plates were spotted with these solutions using two 1 μl aliquots. The plates were developed in acidic or basic solvent and after the silica gel had dried, the acids were detected with basic permanganate solution. The results of silica gel thin-layer chromatography of the acids present in the fractions prepared from the cellulose bands are presented in Table XII.

TABLE XI

CELLULOSE THIN-LAYER SEPARATION OF THE ORGANIC ACIDS OF BRYOPHYLLUM CALYCINUM

Solvent	Spot	R_F Value Range*	Description of Spot	Probable Acid
Acidic	a	23.3-26.4	pink white	
	b	26.1-29.4	pale white	
	c	30.6-33.2	pink brown	NH_4^+ ion
	d	34.3-37.5	medium white	
	e	48.8-50.3	white	Citric+Isocitric + Malic
Basic	a	1.1- 2.2	brown	
	b	6.6- 7.7	white	Citric+Isocitric
	c	11.6-12.8	white	Citric+Isocitric
	d	15.8-17.6	brown-white	
	e	20.3-21.4	white	Malic
	f	23.4-24.7	white	Malic
	g	27.7-30.5	brown-white	
	h	54.5-60.3	very pale white	
	i	62.8-70.1	brown	

* R_F value range represents the maximum difference between plates.

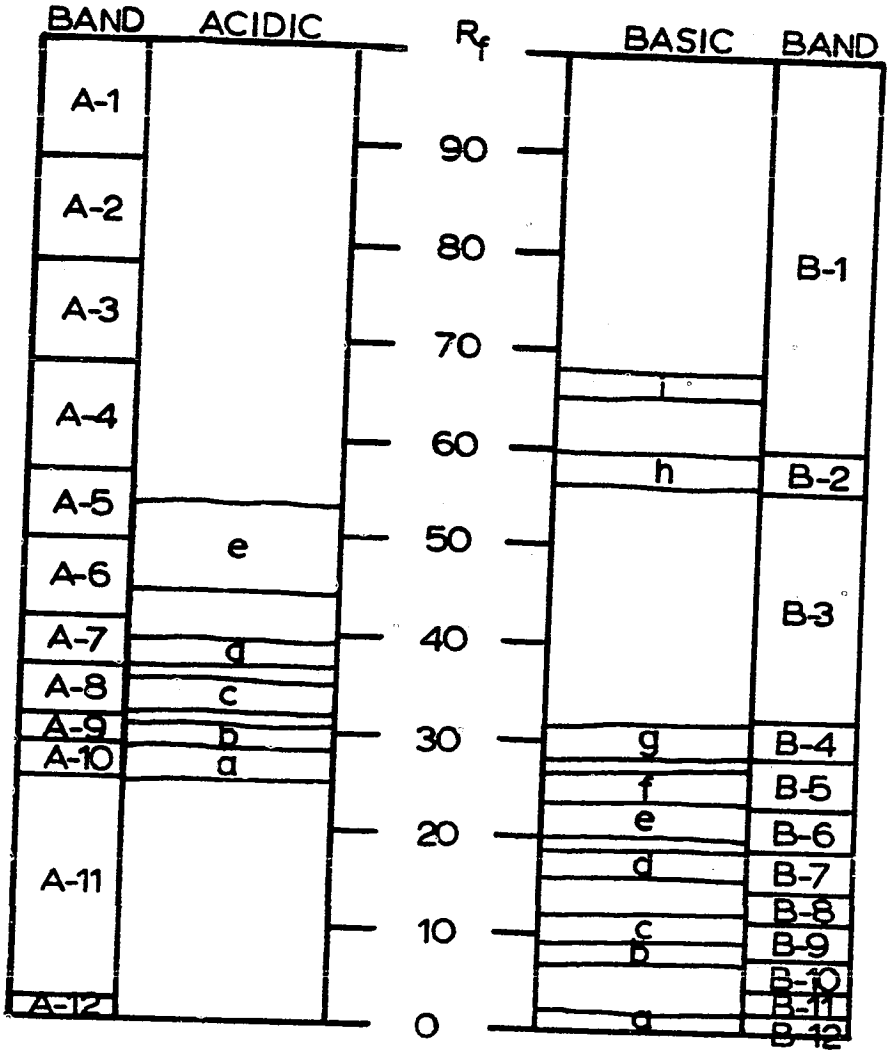


Fig. 4. Preparative cellulose thin-layer separation of the organic acids of Bryophyllum calycinum.

TABLE XII

SILICA GEL THIN-LAYER SEPARATION OF ACIDS EXTRACTED FROM CELLULOSE BANDS

Acidic Solvent				Basic Solvent			
Fraction	R_F Value of Spot	Description of Spot	Probable Acid	Fraction	R_F Value of Spot	Description of Spot	Probable Acid
A-1+A-2	97.4	bright yellow		A-1+A-2			
A-3				A-3			
A-4				A-4	37.8	medium yellow	Malic
A-5	67.2	yellow-white	Malic	A-5	39.0	bright yellow	Malic
A-6	79.2	yellow-white	Malic+Citric +Isocitric	A-6	24.8	bright yellow	Citric+ Isocitric
					43.3	bright yellow	Malic
A-7				A-7			
A-8	72.2	medium yellow		A-8			
A-9				A-9			
A-10				A-10			
A-11				A-11			
A-12				A-12			

TABLE XII (Continued)

Acidic Solvent				Basic Solvent			
Fraction	R_F Value of Spot	Description of Spot	Probable Acid	Fraction	R_F Value of Spot	Description of Spot	Probable Acid
B-1				B-1	79.3	medium yellow	
B-2				B-2			
B-3	30.0	medium yellow		B-3	40.3	bright yellow	Malic
	44.9	medium yellow					
	61.5	medium bright yellow	Malic				
B-4				B-4	41.4	bright yellow	Malic
B-5	62.2	medium yellow	Malic	B-5	41.9	bright yellow	Malic
B-6	63.6	bright yellow	Malic	B-6	36.4	bright yellow	Malic
					43.6	bright yellow	Malic
B-7				B-7	41.0	bright yellow	Malic
B-8				B-8	6.5	medium yellow	Isocitric

TABLE XII (Continued)

Acidic Solvent				Basic Solvent			
Fraction	R_f Value of Spot	Description of Spot	Probable Acid	Fraction	R_f Value of Spot	Description of Spot	Probable Acid
B-9	66.3	medium yellow	Citric+ Isocitric	B-9	9.0	medium bright yellow	Isocitric
					15.7	medium bright yellow	Citric
B-10	71.5	medium yellow	Citric+ Isocitric	B-10	12.4	bright yellow	Isocitric
					22.9	bright yellow	Citric
B-11				B-11	12.8	medium bright yellow	Isocitric
B-12				B-12			

These results correspond to the results obtained on the cellulose plates. Fractions A-1 and A-2 and fraction B-1 apparently contain fast-moving acids since acid spots are detected on silica gel for these fractions. The presence of malic acid as well as citric and isocitric acids in A-6 is indicated by the separation of these acids on silica gel using basic solvent. Malic acid is present in fractions B-3 and B-4 which indicates that some malic acid travels ahead of the main concentration of this acid.

A 1 μl aliquot of each 30 μl fraction was titrated with 0.001 N NaOH to determine the amount of acid present in each fraction and thus the amount of resin necessary for esterification of the acids. The amount of acid in each fraction and the amount of resin added to the acid of each fraction are presented in Table XIII. Since the μeq of acid available for elution from the cellulose were known, the percent recovery of the organic acids from the cellulose plates could be calculated. The percent recovery of the acids is indicated in Table XIII.

The required additional amounts of methanol were added to the resin and methanolic acid solutions contained in culture tubes and the acids were esterified as before using the autoclave. Aliquots of the ester solutions were removed from the supernatant solutions in the culture tubes after they had been allowed to cool. These aliquots were analyzed by gas chromatography using the Carbowax 20 M column. The conditions used in gas chromatography of the esterified fractions are presented in Table XIV.

TABLE XIII

AMOUNT OF ACID IN FRACTIONS PREPARED FROM CELLULOSE BANDS

Fraction	Acid in each Fraction, μeq	Resin Added, mg	Methanol Added, μl	Acid Available for Elution, μeq	Percent Recovery of Acid
A-1+2	2.673	5.6	60		
A-3	5.643	10.8	70		
A-4	6.318	12.3	80		
A-5	34.803	68.3	270		
A-6	65.880	128.9	480		
A-7	25.623	49.9	200		
A-8	2.862	5.3	60		
A-9	5.049	9.8	70		
A-10	3.888	7.7	65		
A-11	7.992	15.8	90		
A-12	0.081	2.2	40		
Total	160.812			181.603	88.6

TABLE XIII (Continued)

Fraction	Acid in each Fraction, μeq	Resin Added, mg	Methanol Added, μl	Acid Available for Elution, μeq	Percent Recovery of Acid
B-1	2.727	5.1	60		
B-2	0.000	2.1	40		
B-3	11.961	24.0	130		
B-4	8.856	17.6	85		
B-5	14.823	29.5	145		
B-6	25.866	49.9	200		
B-7	12.771	25.0	130		
B-8	9.801	18.8	110		
B-9	25.488	50.6	220		
B-10	33.372	65.4	270		
B-11	9.936	18.9	100		
B-12	1.134	2.2	70		
Total	156.735			181.603	86.3

TABLE XIV

CONDITIONS FOR GAS CHROMATOGRAPHY

Sample amount	0.5 μl
Initial temperature	40°C-60°C
Final temperature	215°C
Time for temperature increase	80 min
Oven power	75 and 65
Set to 75	8 min after injection
Set to 65	18 min after injection
Injection block temperature	200°C
Hydrogen flow	25 ml/min
Nitrogen flow	39.0 ml/min
Temperature for nitrogen measurement	19.5°C
Input impedance	10^9
Output sensitivity	10 X
Attenuation	1 X
Range of recorder	1 mv
Chart speed	15"/hr

The results of the gas chromatographic examination of the esterified fractions are presented in Table XV, p. 108, and Figure 5, p. 116.

The retention time of a peak was measured from the apex of the peak to the start of the first peak after injection, which should mark the air peak. Often this peak became a part of the methanol peak. The temperature of elution was considered to be the temperature at which the apex of the peak occurred.

The area of the peaks was determined by weighing the peaks cut from two Xerox copies of graphs drawn from the gas chromatography results. In order to obtain the area of the peak corresponding to the whole fraction, these weights were multiplied by the factor: total volume of solution in the fraction divided by volume of solution injected. These values are those entered in the table and enable a direct comparison between fractions.

Using weight measurements for area depended on the uniformity of Xerox paper. The homogeneity of Xerox paper has been noted previously (30) and the precision of this method for determination of area was found satisfactory. An advantage of this method is that it enables precise determination of the area of irregularly shaped peaks.

The total area of peaks resulting from the "B" fractions was higher than that from the "A" fractions. The areas for malic acid and isocitric acid were both higher in the "B" fractions and contributed the major portion of the difference in area. Estimation of the areas of the peaks representing dimethyl malate and dimethyl isocitric lactone was difficult since attenuation was not used to bring the whole peak

on scale but rather extrapolation was used to plot the peak from which area measurements were made. Cumulative errors in extrapolation are probably the main cause of the difference in total areas of the malic and isocitric ester peaks.

In Table XV, p. 108, the peaks believed to represent the same compound are designated by the same number. In Table XVI these peaks have been arranged in the order of their elution from the Carbowax 20 M column.

Identification of some of these peaks may be made based on the temperature of elution of the ester from the Carbowax 20 M column and the position of the acid on the acidic and basic thin-layer cellulose plates. Peak number 30 represents the dimethyl ester of malic acid. This compound makes up approximately one-third of the acid present, assuming equal response in the flame ionization detector for all acids. This amount of malic acid would be expected in leaves picked during the day when the amount of malic acid has decreased but has not reached its minimum value. Peak 32 represents the dimethyl ester of isocitric lactone. The free isocitric acid is converted entirely to the lactone during the esterification procedure. This acid makes up one-third of the acid present, with no correction being made for differential response of the esters in the detector. Peak 31 represents the trimethyl ester of citric acid and this acid makes up approximately one-tenth of the acid present.

The remaining acids account for approximately 24% of the total acid. This value is higher than would be expected. Authentic

TABLE XVI

ESTERS OF THE ORGANIC ACIDS OF BRYOPHYLLUM CALYCINUM ARRANGED IN ORDER OF THEIR ELUTION FROM CARBOWAX 20 M

Peak Designation	Elution Temperature ("A" Fractions), °C	Elution Temperature ("B" Fractions), °C	"A" Fractions Where Present	"B" Fractions Where Present	Percent of Total Area of "A" Fractions	Percent of Total Area of "B" Fractions	Identity of Acid
1	50	45- 47	12	3- 4	0.09	0.29	
2	53- 55	51	1- 3	1	0.13	0.30	
16		57		6		0.39	
17		64		1		0.09	
3	80- 82	81- 86	9-12	7- 8	1.14	0.96	
33	80- 84	82- 84	1- 5	1- 3	0.63	0.53	Lactic
18		84- 85		11-12		0.08	
35	92	95	11	10	0.17	0.17	Oxalic
34	90- 94	94- 97	1- 4	1- 3	0.38	0.23	Glyoxylic
36	103-105	104-107	1- 5	1- 3	0.85	0.62	
4	104		11		0.42		
40		106		7		0.15	

TABLE XVI (Continued)

Peak Designation	Elution Temperature ("A" Fractions), °C	Elution Temperature ("B" Fractions), °C	"A" Fractions Where Present	"B" Fractions Where Present	Percent of Total Area of "A" Fractions	Percent of Total Area of "B" Fractions	Identity of Acid
37	110-114	114-116	1- 4	1- 4	1.61	2.46	Fumaric + Pyruvic
5*	114-117		9-12		5.42		
19		116		12		0.13	
38	115-118	118-120	1- 4	3- 4	1.08	0.86	Succinic
6	124		4		0.04		
7	130-133	132-134	7-12	1- 4	0.79	0.88	
8	132	132-134	3- 4	7-12	0.31	0.36	
28		136		1		0.15	
29		149		7		0.08	
30	156-160	157-162	3-12	2-12	33.77	31.12	Malic
39*	164-166		7-12		1.43		
20		172-173		1- 2		0.17	
9	175-176	177-180	1- 3	8- 9	1.63	1.36	cis-Aconitic
21		182		1		0.13	
10	185	186	4	11	0.22	0.38	
11*	191		6		1.42		

TABLE XVI (Continued)

Peak Designation	Elution Temperature ("A" Fractions), °C	Elution Temperature ("B" Fractions), °C	"A" Fractions Where Present	"B" Fractions Where Present	Percent of Total Area of "A" Fractions	Percent of Total Area of "B" Fractions	Identity of Acid
31	193	192-194	4- 8	8-12	10.28	8.98	Citric
22		195		1		0.19	
23		197		11		0.20	
12*	199		6- 7		1.80		
24		204		1		0.40	
13*	204		3		2.28		
14	208	209	10	3- 4	0.76	0.91	
25*		209-210		5- 7		8.77	
32	208-211	209-213	3-12	8-12	32.36	32.60	Isocitric
26*		211-212		1- 4		2.20	
15	214	211-212	4	6- 7	1.04	1.26	
27*		211-212		11-12		2.56	

compounds should be apparent on both sets of chromatograms ("A" fractions and "B" fractions). In some cases where the amount of acid is quite small, it may be missed on one or the other set of chromatograms. This apparently occurred with peaks 16, 17, 18, 4, 40, 19, 6, 28, 29, 20, 21, 22, 23, and 24. However, where the amount of the compound detected on one set of chromatograms is large, it should be present on the other set. If it fails to appear, that compound may be assumed to be an artifact resulting from the solvent or the cellulose or a combination of both. Peaks which are believed to represent artifact compounds are 5, 39, 11, 12, 13, 25, 26, and 27. These are marked with asterisks in Table XVI. If the percent areas for these peaks are subtracted from the approximately 24% representing acids other than malic, citric, and isocitric acids, the resulting amount of approximately 11% representing authentic acids is in line with expected values (107).

The identification of some of the acids other than citric, isocitric, and malic acids was made from the position of the acids on the cellulose thin-layer chromatograms and from the elution temperature of their esters on the Carbowax 20 M column. The identifications proposed for these acids are presented in Table XVI and the percentages of the total acid content that these acids represent are also indicated. The percentages of oxalic, glyoxylic, and lactic acids with respect to the total acid content may be higher than those indicated since a portion of these acids may have been lost when the aqueous solutions of acids were dried prior to esterification. Although the esters of fumaric and pyruvic acids are eluted at the same temperature, the distribution of the percent areas for this peak between fractions

suggests that fumaric and pyruvic acids are present in nearly equal amounts and that each represents approximately 1% of the total acid content. Succinic acid also apparently represents about 1% of the acid content and this is in agreement with the expected amount of this acid (15). Cis-aconitic acid, which was found to make up approximately 1.5% of the acid content, would be expected in leaf tissue where iso-citric acid is present in such large amounts and variation occurs in the level of citric acid.

In addition to these acids, a number of unidentified acids contribute to the total percentage of the minor acid fraction. Among those which contribute significantly to this percentage are the acids represented by peaks 3, 36, 7, and 8.

G. Large Scale Separation of the Organic Acids of Bryophyllum calycinum

In order to obtain the organic acids of Bryophyllum calycinum in larger amounts so that their identity could be determined, a large scale extraction of dried leaf tissue was carried out. In addition, in order to further separate the minor and major acids, a preliminary fractionation of the extract was performed using gradient elution from a carbonate form anion exchange column.

Twenty grams of dried leaf tissue were extracted with water in the presence of cation exchange resin as described previously and the total extract was passed onto a column containing anion exchange resin in the carbonate form. After neutral materials had been removed, the acids were eluted from the column with an eluant of increasing $(NH_4)_2CO_3$ concentration and 200 fractions of acid were collected. The gradient of ammonium carbonate concentration used is presented in Figure 6.

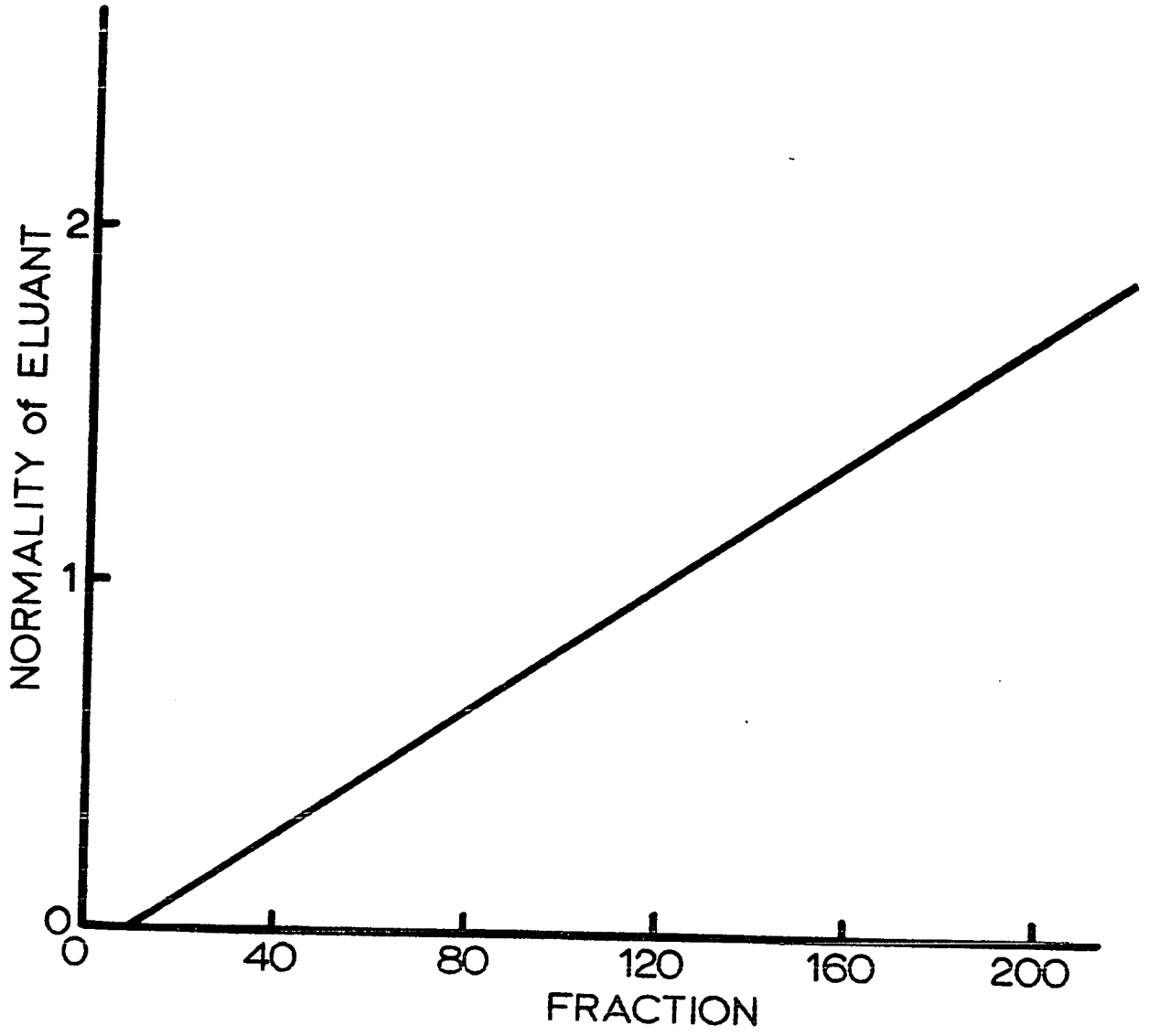


Fig. 6. Gradient of ammonium carbonate concentration used in the elution of the organic acids of Bryophyllum calycinum from a carbonate form anion exchange resin column.

The manner in which the collected acid fractions were to be pooled was determined by the results of silica gel thin-layer chromatography, in both acidic and basic solvents, of the acid contained in 1 ml of every third fraction. The results of the separation of the acids on silica gel plates are presented in Figure 7. The collected fractions were pooled as illustrated in Figure 7 and as outlined in Table XVII.

TABLE XVII

POOLING OF ACID FRACTIONS COLLECTED FROM THE CARBONATE FORM ANION EXCHANGE COLUMN

Designation for Pooled Fraction	Collected Fractions Pooled
A	1 - 14
B	15 - 27
C	28 - 42
D	43 - 61
E	62 - 95
F	96 - 119
G	120 - 140
H	141 - 161

Pooled fractions A, B, C, and D should contain weaker acids and many of the mono- and dicarboxylic minor acids of interest. From the silica gel thin-layer chromatography results, it appeared that fraction E should contain the majority of malic acid; fractions F and G, the majority of citric and isocitric acids. In these fractions

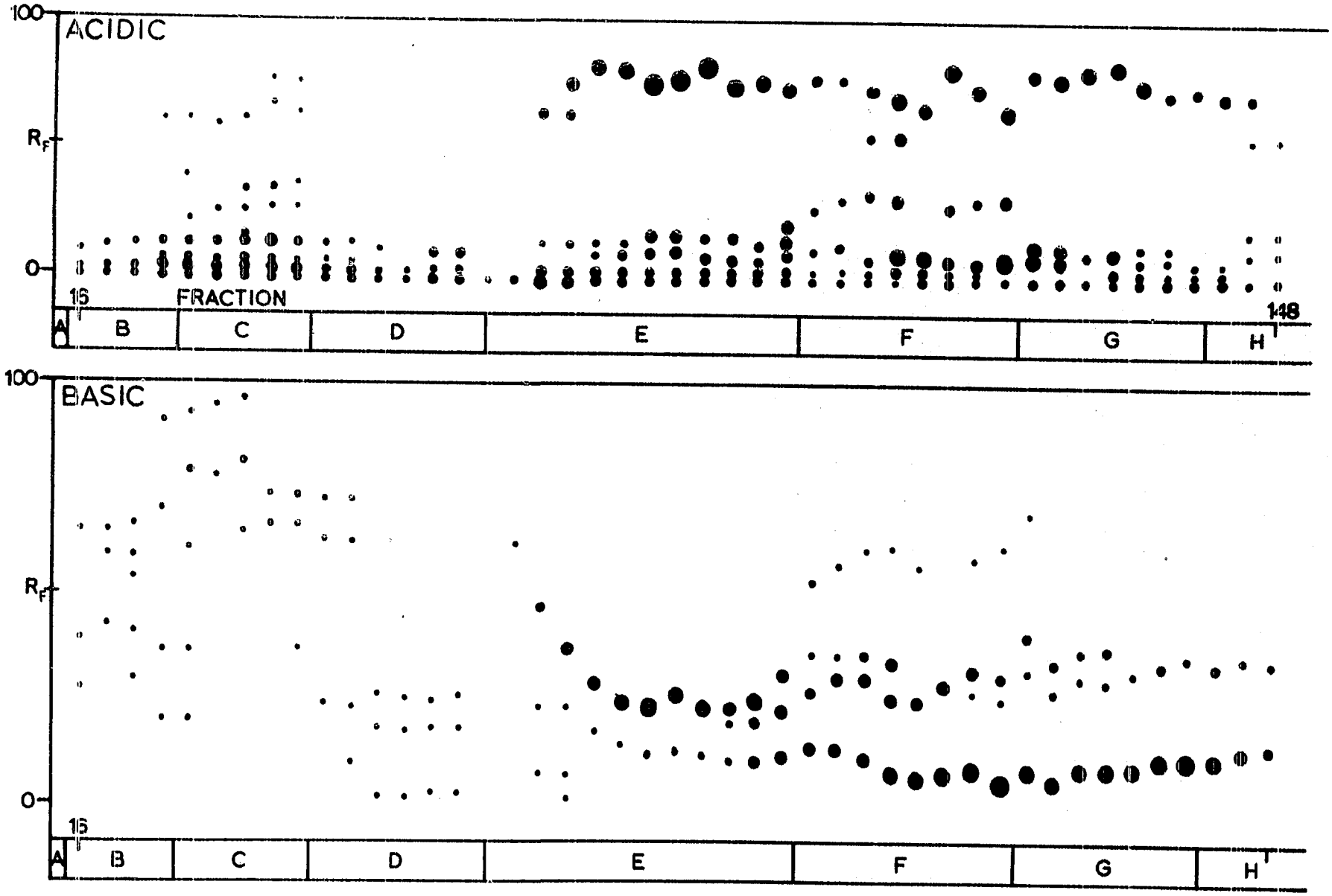


Fig. 7. Silica gel thin-layer separation of the organic acids collected from the carbonate form anion exchange column. Size of spot is proportional to amount of acid.

will occur stronger dicarboxylic acids and tricarboxylic acids.

The pooled fractions were dried in vacuo at 60°C at which temperature $(NH_4)_2CO_3$ is decomposed and removed as ammonia and CO_2. Successive 5 ml solutions were prepared from each fraction by adding a small amount of water to the acid residue, dissolving as much acid as possible, and transferring this concentrated solution to a 5 ml volumetric flask. This process was repeated until 5 ml were collected at which time a new solution was started. This allowed the concentration of the majority of the acid in the least possible volume. The successive solutions prepared from each fraction were labelled A-i; B-i, B-ii; C-i, C-ii, etc. In order to determine the amount of acid present in each solution, small aliquots of each solution were titrated with 0.02 N HCl to the acid end-point of the thymol blue present in the thymol blue-phenolphthalein indicator. The amount of solution used ranged from 0.1 ml to 0.01 ml depending on the amount of acid presumed to be present in the solution. Table XVIII shows the percentage of the total acid present in each solution and those solutions which contained more than 40% of the acid in that fraction are indicated. The percentage of the total acid in each fraction is in agreement with the results of silica gel thin-layer chromatography of the collected fractions.

Those solutions which had more than 40% of the acid of each fraction were spotted as bands on the number of sheets of Whatman # 17 chromatography paper indicated in Table XVIII. These chromatograms were developed in the acidic solvent; ether : formic acid : water, 5 : 2 : 1. After the paper had dried and the formic acid had evaporated,

TABLE XVIII

DISTRIBUTION AMONG POOLED FRACTIONS OF ACIDS ELUTED FROM CARBONATE FORM ANION EXCHANGE COLUMN

Solution	Percent of Total Acid	Percent of the Acid in the Fraction	Number of Chromatogram Sheets Used
A-i	0.008	100.0	1
B-i	1.16	86.8	2
B-ii	0.17		
C-i	2.11	98.5	2
C-ii	0.02		
D-i	1.74	95.3	2
D-ii	0.07		
D-iii	0.05		
D-iv	0.00		
E-i	34.54	88.6	12
E-ii	3.44		
E-iii	0.63		
E-iv	0.08		

TABLE XVIII (Continued)

Solution	Percent of Total Acid	Percent of the Acid in the Fraction	Number of Chromatogram Sheets Used
F-i	16.88	51.5	6
F-ii	14.36	45.5	6
F-iii	0.95		
F-iv	0.02		
G-i	20.06	90.3	10
G-ii	1.94		
G-iii	0.13		
H-i	1.47	92.3	2
H-ii	0.18		

the chromatograms were sprayed with a solution of bromphenol blue which allowed detection of the acids as yellow spots on a blue background. The results of the paper chromatographic separation of the acids of each fraction are presented in Figure 8 and Table XIX. The relative intensity of the spots was represented by + signs and ranged from pale yellow (+) to bright yellow (+++++). The horizontal guide lines for each fraction represent the divisions made of the chromatograms of each fraction prior to elution. The number between the lines is the number which was given to the acid solution resulting from the elution of that section of the chromatogram.

The unsprayed regions of the chromatograms were cut into the horizontal bands indicated and the acids in the corresponding bands of a fraction were collected by downward elution of the paper with 3 N NH_4OH. These sub-fractions were given the numbers of the eluted bands and were dried.

The amount of acid present in each sub-fraction was estimated from the results in Table XVIII and from the appearance of the paper chromatograms after detection of the acids. The acid in each sub-fraction or, where the amount of acid was large, a portion of the acid in each sub-fraction was transferred to a culture tube of 8 ml capacity and the acid solution was taken to dryness using an air stream. The appropriate amount of resin for the esterification of the amount of acid assumed to be present was added to these tubes, followed by sufficient methanol to give an excess of 25 to 50 μl. The acids were esterified in the autoclave as described previously.

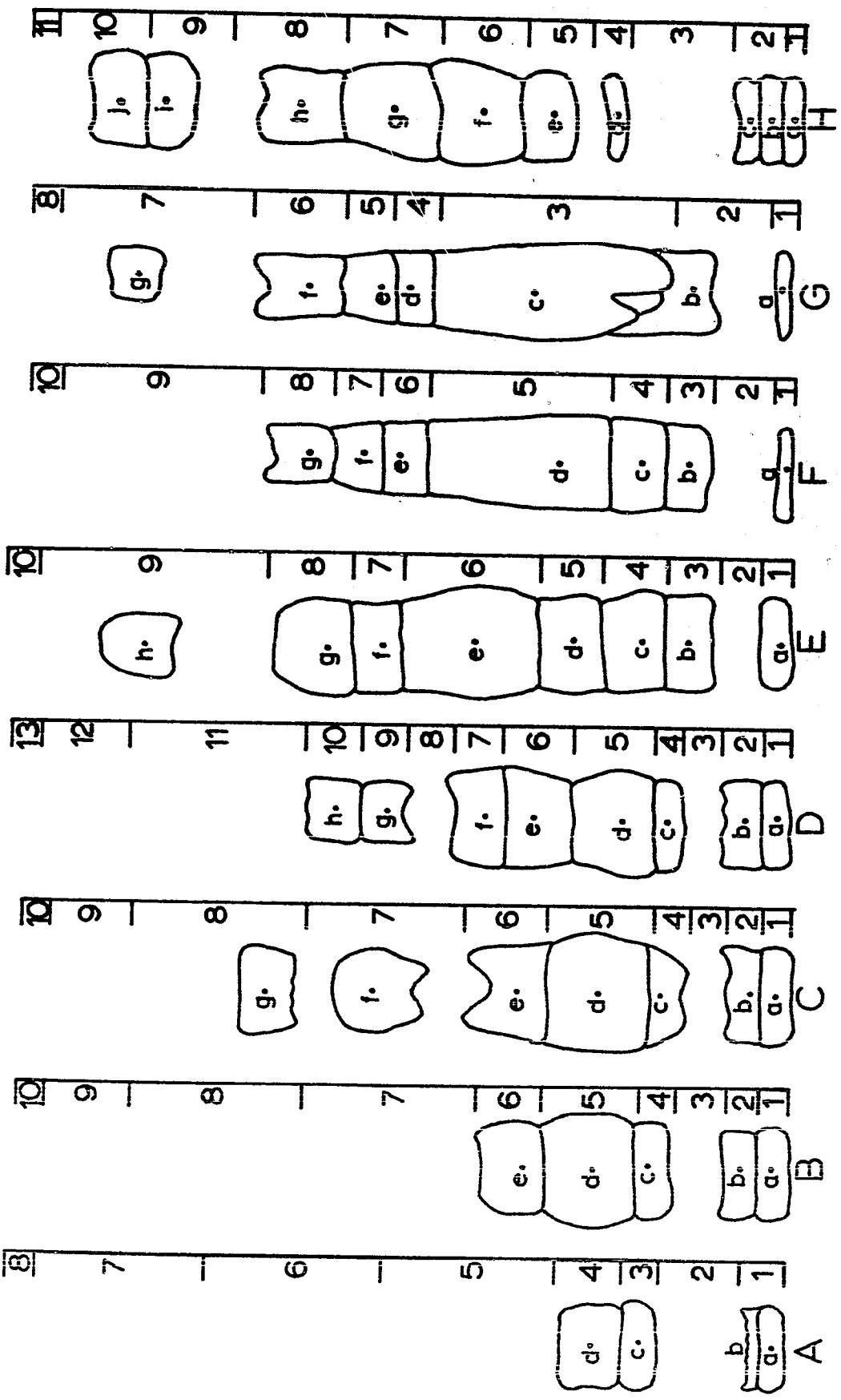


Fig. 8. Separation of the organic acids in pooled fractions on paper chromatograms

TABLE XIX

SEPARATION OF THE ORGANIC ACIDS OF BRYOPHYLLUM CALYCINUM ON PAPER CHROMATOGRAMS

Spot	R_f Value Range	Intensity
A-a	0.0	+++
A-b		
A-c	17.3	+
A-d	23.6	++
B-a	0.0	+++
B-b	4.3 - 4.5	+
B-c	15.3 - 16.4	++
B-d	22.0 - 23.6	+++
B-e	31.4 - 32.9	++
C-a	0.0	++
C-b	3.2 - 3.4	+
C-c	14.4 - 15.7	++
C-d	22.4 - 23.8	+++
C-e	32.5 - 34.5	++
C-f	52.6 - 53.3	++
C-g	67.8	++
D-a	0.0	+
D-b	4.0 - 4.7	+
D-c	14.5 - 16.1	+
D-d	20.2 - 22.2	+++
D-e	31.6 - 34.2	++

TABLE XIX (Continued)

Spot	R_f Value Range	Intensity
D-f	37.9 - 39.8	++
D-g	50.7 - 53.5	+
D-h	57.8 - 59.9	+
E-a	0.0	+
E-b	11.2 - 13.3	+++
E-c	18.0 - 20.6	++
E-d	25.4 - 28.7	++
E-e	37.0 - 41.4	++
E-f	49.7 - 53.2	+++++
E-g	56.8 - 59.1	+++++
E-h	80.9 - 83.3	+
F-a	0.0	+
F-b	10.1 - 13.6	++
F-c	16.0 - 19.0	++
F-d	27.5 - 32.1	+++
F-e	48.6 - 53.2	+++
F-f	54.0 - 57.5	+++
F-g	60.8 - 64.2	+

TABLE XIX (Continued)

Spot	R_F Value Range	Intensity
G-a	0.0	+
G-b	10.4 - 13.3	+
G-c	29.2 - 35.8	+++
G-d	48.5 - 50.9	++++
G-e	52.9 - 55.8	+++
G-f	62.1 - 64.6	+
G-g	85.7 - 87.6	+
H-a	0.0	+++
H-b	2.6	+
H-c	5.1	+
H-d	22.7 - 22.9	+
H-e	30.9 - 31.0	+
H-f	40.3	++
H-g	52.3 - 53.1	+++
H-h	64.2 - 64.3	+
H-i	81.5 - 82.3	++
H-j	87.7 - 87.8	+++

Small aliquots of the supernatant ester solution were removed from each tube and analyzed by analytical gas chromatography at oven temperatures of 100°, 150°, and 210°C using the Carbowax 20 M column. The conditions used for gas chromatography of the esterified fractions on the Carbowax column are presented in Table XX.

TABLE XX

CONDITIONS FOR GAS CHROMATOGRAPHY OF METHYL ESTERS ON CARBOWAX 20 M

Sample amount	0.5 µl
Oven temperature	100°, 150°, 210°C
Injection temperature	200°C
Hydrogen flow	30 ml/min
Nitrogen flow	57.1 ml/min
Temperature for nitrogen measurement	20.5°C
Input impedance	10^9
Output sensitivity	1 X
Attenuation	1 X
Range of recorder	1 mv
Chart speed	15"/hr

The results of the gas chromatographic examination of the esters on the Carbowax column are presented in Table XXI, p. 129. The retention volumes for these esters were calculated in the same manner as for the standard esters. The esters determined in each fraction were designated by letters; a, b, c, d, etc.

The remaining supernatant solution of each esterified fraction was injected into the A-100 preparative gas chromatograph where the esters were separated by programmed temperature heating of the silicone column. The conditions used for gas chromatography of the esterified fractions on the silicone SE-30 preparative column are presented in Table XXII.

TABLE XXII

CONDITIONS FOR GAS CHROMATOGRAPHY OF METHYL ESTERS ON SE-30 SILICONE

Sample amount	25 - 50 µl
Initial oven temperature	57 - 84°C
Final oven temperature[a]	220°C
Time for temperature increase	60 min
Power setting for temperature increase	100
Injection temperature	175 - 200°C
Helium flow	40 ml/min
Sensitivity	maximum
Recorder range	1 mv
Chart speed	15"/hr

[a] Temperature was maintained at 220°C for 10 min by setting the power to 78.

The temperatures of elution of the esters from the silicone column are also presented in Table XXI, p. 129.

Although analyzed, no results were obtained from the A-solutions so the results in Table XXI begin with the B-solutions.

The fractions at the solvent front were not examined since material from the paper was apparently concentrated in this region. It was not likely that any acid of interest would have an R_F value in this region.

The G- and H- solutions were examined separately on the Carbowax 20 M column but were combined as follows for examination on the silicone column and subsequent infrared spectroscopy:

G-1 + H-1	=	GH-1
G-2 + H-2 + H-3	=	GH-2
G-3 + H-4 + H-5 + H-6	=	GH-3
G-4 + G-5 + H-7	=	GH-4
G-6 + H-8	=	GH-5
G-7 + H-9 + H-10	=	GH-6

Each peak in Table XXI, p.129, was designated by a number and peaks which are believed to represent the same compound have the same number. In Table XXIII the numbered peaks have been arranged in the order of their elution from the Carbowax 20 M column. The temperature at which the compound was eluted from the preparative silicone column and the fractions in which the compound occurred are also listed. The correlation of the Carbowax elution peaks with the silicone elution peaks was carried out by comparison of the two sets of chromatograms.

The esters obtained from the esterified acid fractions were collected in glass capillary tubing as they were eluted from the silicone column. The esters were then dissolved in a small amount of chloroform and transferred to infrared microcells. These solutions were examined in a double beam infrared spectrophotometer using a chloroform blank in the reference beam. In Figure 9, p.139, are

TABLE XXIII

METHYL ESTERS OF THE ORGANIC ACIDS OF BRYOPHYLLUM CALYCINUM ARRANGED IN ORDER OF THEIR ELUTION FROM CARBOWAX 20 M

Peak	Retention Volume on Carbowax, ml 100°C	150°C	210°C	Elution Temperature from Silicone, °C	Fractions in which Ester is Present B	C	D	E	F	G	H	Acid
7	51.3- 55.6				3- 6							
6	51.3- 55.6			103	7	7	8					
39	55.6- 59.9			108					7- 8			
62	59.9										4- 5	
68	51.3- 64.1			96-103	8- 9	8- 9	10-12	9	9	5- 7	6-10	Lactic
23	51.3- 64.1						5	4- 5	3- 4			
24	55.6- 64.1			102-109			2- 3	1- 2	1- 2	1- 2	1- 3	
15	77.0			110		8						
2	77.0- 81.2			88	8	7- 8	11	9	9			Glyoxylic
51	81.2- 89.8			114-117						5- 7	8-10	
64	85.5- 94.1									1- 2	1- 2	
1	89.8- 94.1			112-114	9	9	12					
63	94.1										4- 5	
20	94.1			108			12					
67	85.5- 98.3			108-116	2- 6	3- 6	2- 6	3- 5	1- 4			Oxalic
32	98.3			116				1				

TABLE XXIII (Continued)

Peak	Retention Volume on Carbowax, ml 100°C	150°C	210°C	Elution Temperature from Silicone, °C	Fractions in which Ester is Present B	C	D	E	F	G	H	Acid
38	102.6								9			
22	89.8-111.2			118			8-10	6				
41	132.5-145.4			138					5	3		
42	145.4			110					5			
26	149.6							8				
59	158.2									6		
54	158.2-171.0			131							8-10	
72	171.0			136				9				
17	200.9			127		8						
16	200.9			120		8						
3	183.8-205.2			136-142	8	8- 9	11					
12	205.2				2							
28	205.2-213.8			152				8	8			
53	205.2-218.0									6- 7	9	
58	243.7			133						5		
47	256.5								2			

TABLE XXIII (Continued)

Peak	Retention Volume on Carbowax, ml 100°C	150°C	210°C	Elution Temperature from Silicone, °C	Fractions in which Ester is Present B	C	D	E	F	G	H	Acid
52	265.1-273.6			148						6- 7	8-10	
4	239.4-282.2			148-151	8	8- 9	11-12	8- 9	8- 9			Succinic
65	303.5-316.4									2	3	
55	414.7										10	
25		68.9		158				9				
60		83.4									9	
44		83.4- 97.9		168-171					4- 5	3		
43		97.9		157					5			
13		145.0		163		8						
48		184.9		148					2			
19		195.8		158		7						
18		192.1-206.6		133		5- 7						
69		199.4-235.6		158-166			8-11	1- 9	4- 8		7-10	Malic
8		217.5-242.9		161-163	3- 5	2- 4						
36		261.0-268.3						1- 2				
73		290.0							2			

TABLE XXIII (Continued)

Peak	Retention Volume on Carbowax, ml 100°C	150°C	210°C	Elution Temperature from Silicone, °C	Fractions in which Ester is Present B	C	D	E	F	G	H	Acid
66		297.3						9				
14			51.9	171		8						
11			61.0		2							
27			64.1					9				
56			64.1- 67.1	193							9-10	cis-Aconitic
34			91.5					1				
10			91.5	195	2							
21			91.5-100.7				12	9				
70			106.8-125.1	198-202				5- 7	4- 7	4- 5		Citric
61			152.5							3		
35			167.8					1				
37			164.7-183.0						9	6- 7		
5			195.2-207.4	196	8	7- 8	11					
50			210.5						2			
29			228.8	197				7				
71			228.8-256.2	198-206				5- 7	3- 8	3- 6	5- 8	Isocitric

TABLE XXIII (Continued)

Peak	Retention Volume on Carbowax, ml 100°C	150°C	210°C	Elution Temperature from Silicone, °C	Fractions in which Ester is Present B	C	D	E	F	G	H	Acid
45			250.1-253.2	199					1	1		
9			256.2		6							
33			256.2					3				
46			286.7	220					5			
57				150					4			
49				178-181					4- 6	4- 6	7- 8	
40				171					4			
31				204				3				
30				206				6				

presented the infrared spectra of the compounds obtained from the esterified acid fractions. The spectra of these esters are labelled both by the fraction and ester designation and by the peak number designation. The infrared spectra of these esters were for the most part less distinct than those of known esters since a smaller amount of ester was obtained.

Known esters were prepared by esterification of the acids as described previously and these esters were collected in capillary tubing after analysis by preparative gas chromatography using the silicone column. The esters were dissolved in chloroform and analyzed by infrared spectroscopy. In Figure 9, p. 139, are presented the infrared spectra of these known esters. These spectra indicate that if sufficient ester is present it is possible to distinguish one ester from another. These spectra show basic similarities as would be expected from the similar structure of the molecules. The region which in the main allows differentiation of the molecules lies above 7.0 microns. In addition, the absorption between 6.0 and 6.1 microns shown by dimethyl fumarate and trimethyl cis-aconitate indicates the presence of the double bond in these molecules. The lactone ring of dimethyl isocitric lactone is indicated by the absorption at 5.5 - 5.6 microns. The hydroxyl groups of lactic, malic, tartaric, and citric methyl esters are indicated at 2.83 - 2.85 microns.

The identification of individual acids giving rise to particular peaks was based on the retention volume of the ester on Carbowax 20 M, the elution temperature of the ester from silicone, the time of emergence of the acid from the ion exchange resin, the

position of the acid on the paper chromatograms, and the match between the infrared spectrum of the isolated ester and the infrared spectrum of the known ester. These identifications of acids are presented in Table XXIII.

The dimethyl ester of fumaric acid, which was determined in the previous experiment, is not apparently represented by any of the peaks in the present experiment. This would seem to indicate that the elution of fumaric acid from the carbonate form resin column occurs later than the elution of other acids. The elution of fumaric acid from a formate column using formic acid as eluant occurs after citric acid (66). Although the fractions collected from the carbonate column after those which were pooled to give fraction H appeared to contain no acid, as determined by silica gel thin-layer chromatography, they were pooled and dried in vacuo. This dried pooled fraction, containing any acids present in fractions 162 to 210, was dissolved in water and spotted as described previously on Whatman # 17 paper. The chromatogram was developed in ether : formic acid : water (5 : 2 : 1), dried, and spraved with a solution of bromphenol blue to detect the acids. In addition to other acid spots, a spot was observed at R_f 96.6 which was believed to represent fumaric acid. The reason fumaric acid was not detected in silica gel thin-layer chromatograpny of the original late fractions may have been that fumaric acid came off as a broad peak from the carbonate form resin column rather than as a sharp peak. This would result in a low concentration of fumaric acid in all fractions and prevent its detection.

The presence of pyruvic acid in Bryophyllum leaf tissue was determined in the previous experiment. In the present experiment, no ester was eluted from both Carbowax and silicone columns at the same position as the ester prepared from pyruvic acid. However, peak 3 is believed to represent the ester of pyruvic acid. The acid giving rise to peak 3 had an R_F value on paper similar to that for pyruvic acid. As well, the ester prepared from this acid had a retention volume on the Carbowax column the same as that for the ester prepared from pyruvic acid. Finally, the spectrum of this unknown ester matched the spectrum of the ester of pyruvic acid, although both spectra were prepared using small amounts of ester. However, the elution temperature of this ester from the silicone column was only 136 - 142°C, whereas that for the ester of pyruvic acid was 153°C. The lower elution temperature would seem more reasonable for the ester of pyruvic acid, considering its retention volume on the Carbowax column. The reason for the discrepancy in temperatures of elution between the two apparently identical molecules is not known.

Peak 14 is believed to represent the ester of α-ketoglutaric acid. The R_F value of the acid giving rise to this peak was similar to that of α-ketoglutaric acid. The ester was eluted from the silicone column at the same temperature as dimethyl α-ketoglutarate. The retention volume of the ester on the Carbowax column was larger than that for dimethyl α-ketoglutarate and was close to the retention volume that would be expected for the enol form of dimethyl α-ketoglutarate. The infrared spectrum of the unknown ester was identical to that of dimethyl α-ketoglutarate except at higher wavelengths and

included the shoulder at a lower wavelength observed on the main C=O absorption.

Peak 19 is believed to represent the dimethyl ester of the enol form of oxalacetic acid. This derivative was not determined in the previous esterifications of oxalacetic acid but since oxalacetic acid is thought to be considerably enolized, the production of the enol ester on esterification would be expected. The R_F value of this acid was similar to the R_F value expected for the enol form of oxalacetic acid. The ester of this acid was eluted from the silicone column at the temperature at which the ester of oxalacetic acid should be eluted and the retention volume of the ester on Carbowax was similar to the retention volume that the oxalacetate ester should have. The infrared spectrum of the unknown ester indicated the presence of a hydroxyl group in the molecule and possibly $\alpha:\beta$ unsaturation.

Tentative characteristics of the remaining acids may be determined from the results in Table XXIII.

In most cases, the strength of the acid should be indicated by its time of emergence from the carbonate form ion exchange column. Those acids collected in pooled fractions A, B, C, and D may be considered acids of weak to medium strength. Those acids collected in the remaining fractions represent the medium strength to strong acids. The acids of greatest interest are the weak and medium strength acids since these correspond best to the known acids involved in crassulacean acid metabolism and the Krebs cycle.

In the paper chromatographic separation of the organic acids, formic acid is present as a component of the developing solvent. This

acid acts as a "swamp" acid and represses the ionization of the organic acids. Thus, the functional groups present in the acid molecule, rather than its pK_a, have the major influence on the distance the acid will travel. The R_f of the acid provides an indication of the number and type of functional groups present in the molecule.

The temperature at which an ester is eluted from the silicone column is indicative of the boiling point of the ester and gives an idea as to the chain length of the molecule and the number of carboxyl groups present.

The boiling point of the ester may also be deduced from the retention volume of the ester on the Carbowax 20 M column. However, if polar functional groups are present in the molecule, the elution of the ester will be retarded and the apparent boiling point will be higher than the actual boiling point of the ester. Thus, if the boiling points determined by elution of the ester from the silicone and Carbowax columns are similar, there is probably little retardation in elution of the ester from the Carbowax column by functional groups in the molecule. If the boiling point determined by the elution of the ester from the Carbowax column is higher than that determined using the silicone column, it indicates that the ester is probably retarded in its elution from the Carbowax column by the presence of polar functional groups in the molecule.

The infrared spectrum of the ester molecule allows some information to be obtained with regard to the structure of the ester and the presence of functional groups. Since the spectra of esters

of carboxylic acids are similar, elucidation of the structure from a recognition of the absorptions due to specific groups or arrangements of atoms is difficult. Identification is thus best carried out by a comparison of the spectrum of the unknown ester with the spectra of esters of known acids.

Using these criteria, it is possible to elucidate tentative characteristics for the uncharacterized acids whose esters form the unidentified peaks. The results of this study are presented in Table XXIV.

TABLE XXIV

TENTATIVE CHARACTERISTICS OF UNIDENTIFIED ACIDS OF BRYOPHYLLUM CALYCINUM

Peak	Acid Chain Length, Number of C	Number of Carboxyl Groups	Presence of Other Groups	Boiling Point of Methyl Ester, °C
7	3	1	yes	140
6	3	1	yes-OH	145
39	4	1	yes	155
62	3	1	yes	145
23	3	1	yes	145
24	4	1	yes-OH	155
15	4	1	yes	160
51	4	1	yes	165
64				
1	4	1	yes	165
63	4	1	yes	155
20	4	1	yes	157
32	4	1	yes	170
38	4	1	yes	176
22	4	1	yes	175
41	3	2	yes	185
42	6	1	no	162
26	3	1	yes	177
59	3	1	yes	177
54	3	2	yes	183
72	5	1	yes	188
17	5	1	yes	180

TABLE XXIV (Continued)

Peak	Acid Chain Length, Number of C	Number of Carboxyl Groups	Presence of Other Groups	Boiling Point of Methyl Ester, °C
16	6	1	no	173
12				
28	5	1	yes	195
53	4	2	yes	190
58	4	1	yes	185
47				
52	4	2	yes	194
65				
55	5	2	no	210
25	5	2	no	220
60	5	2	yes	225
44	5	2	yes	265
43	3	2	yes-OH	225
13	4	2	yes	240
48	3	1	yes	195
18	3	1	yes-OH	185
8	4	2	yes-OH	240
36	3	1	yes	240
73	3	1	yes	240
66	5	2	yes	270
11				
27	6	3	no	270

TABLE XXIV (Continued)

Peak	Acid Chain Length, Number of C	Number of Carboxyl Groups	Presence of Other Groups	Boiling Point of Methyl Ester, °C
34				
10	6	3	no	280
21	6	3	no	280
61	4	2	yes	280
35	4	2	yes	280
37	5	2	yes	295
5	6	3	no	285
50	3	2	yes	280
29	6	3	yes	290
45	6	3	yes	290
9	5	2	yes	290
33	5	2	yes	290
46	7	3	yes	310
57	4	1	yes	200
49	5	2	yes	270
40	5	2	yes	260
31	6	3	yes	290
30	6	3	yes	293

TABLE XV

GAS CHROMATOGRAPHY OF THE METHYL ESTERS OF THE ORGANIC ACIDS OF BRYOPHYLLUM CALYCINUM

Peak	Time, min	Temp, °C	Area	Percent of Total Area of "A" Fractions	Peak Designation
A-1+2-a	8.80	55	1.136	0.08	2
A-1+2-b	14.16	84	1.840	0.13	33
A-1+2-c	16.32	90	2.448	0.18	34
A-1+2-d	18.72	103	1.440	0.10	36
A-1+2-e	21.08	110	6.144	0.45	37
A-1+2-f	22.72	115	1.952	0.14	38
A-1+2-g	49.16	175	20.304	1.47	9
Total			35.264	2.55	
A-3-a	9.52	53	0.636	0.05	2
A-3-b	16.96	80	1.387	0.10	33
A-3-c	19.80	94	1.527	0.11	34
A-3-d	22.20	104	1.795	0.13	36
A-3-e	24.80	114	5.294	0.38	37
A-3-f	26.36	118	5.256	0.38	38
A-3-g	31.60	132	1.756	0.13	8
A-3-h	42.60	156	10.309	0.75	30
A-3-i	53.12	176	2.164	0.16	9
A-3-j	74.88	204	31.448	2.28	13
A-3-k	79.96	209	24.716	1.79	32
Total			86.288	6.26	

TABLE XV (Continued)

Peak	Time, min	Temp, °C	Area	Percent of Total Area of "A" Fractions	Peak Designation
A-4-a	17.20	84	1.664	0.12	33
A-4-b	19.28	94	1.216	0.09	34
A-4-c	21.60	103	4.816	0.35	36
A-4-d	23.80	113	10.752	0.78	37
A-4-e	25.28	118	7.680	0.56	38
A-4-f	27.28	124	0.608	0.04	6
A-4-g	30.20	132	2.512	0.18	8
A-4-h	40.44	159	30.416	2.20	30
A-4-i	52.28	185	3.088	0.22	10
A-4-j	57.88	193	4.032	0.29	31
A-4-k	70.20	211	37.888	2.75	32
A-4-l	72.64	214	14.352	1.04	15
Total			119.024	8.62	
A-5-a	14.48	82	3.840	0.28	33
A-5-b	19.32	105	3.660	0.27	36
A-5-c	36.04	160	270.660	19.62	30
A-5-d			7.620	0.55	32
Total			285.780	20.72	
A-6-a	43.36		113.966	8.26	30
A-6-b	59.20	191	19.611	1.42	11
A-6-c	61.04		99.292	7.20	31
A-6-d	64.72	(199)	21.943	1.59	12
A-6-e			186.387	13.51	32
Total			441.199	31.98	

TABLE XV (Continued)

Peak	Time, min	Temp, °C	Area	Percent of Total Area of "A" Fractions	Peak Designation
A-7-a	32.88	132	3.600	0.26	7
A-7-b	43.96	160	33.550	2.43	30
A-7-c	47.04	166	17.050	1.24	39
A-7-d	62.60	193	36.650	2.66	31
A-7-e	67.28	199	2.850	0.21	12
A-7-f	76.20	210	112.650	8.17	32
Total			206.350	14.97	
A-8-a	31.28	133	2.304	0.17	7
A-8-b	60.48	193	1.738	0.13	31
A-8-c	75.64	210	16.547	1.20	32
Total			20.589	1.50	
A-9-a	15.00	80	4.107	0.30	3
A-9-b	23.24	117	58.942	4.27	5
A-9-c	38.80	159	0.918	0.07	30
A-9-d	71.72	208	38.999	2.83	32
Total			102.966	7.47	
A-10-a	17.12	81	7.323	0.53	3
A-10-b	24.88	117	3.835	0.28	5
A-10-c	30.44	133	1.462	0.11	7
A-10-d	41.72	159	1.235	0.09	30
A-10-e	45.04	166	0.661	0.05	39
A-10-f	73.80	208	10.519	0.76	14
A-10-g	76.96	210	13.227	0.96	32
Total			38.262	2.78	

TABLE XV (Continued)

Peak	Time, min	Temp, °C	Area	Percent of Total Area of "A" Fractions	Peak Designation
A-11-a	18.28	82	3.348	0.25	3
A-11-b	21.12	92	2.376	0.17	35
A-11-c	23.04	104	5.760	0.42	4
A-11-d	25.56	114	10.026	0.73	5
A-11-e	31.56	132	2.772	0.20	7
A-11-f	42.64	159	3.906	0.28	30
A-11-g	45.44	164	1.548	0.11	39
A-11-h	76.96	210	5.220	0.38	32
Total			34.956	2.54	
A-12-a	11.04	50	1.262	0.09	1
A-12-b	18.20	81	0.880	0.06	3
A-12-c	25.52	114	1.884	0.14	5
A-12-d	31.04	130	0.631	0.05	7
A-12-e	42.28	160	0.898	0.07	30
A-12-f	45.20	165	0.382	0.03	39
A-12-g	76.60	211	2.978	0.22	32
Total			8.915	0.66	
Total of "A" fractions			1379.593		

TABLE XV (Continued)

Peak	Time, min	Temp, °C	Area	Percent of Total Area of "B" Fractions	Peak Designation
B-1-a	10.72	51	4.992	0.30	2
B-1-b	14.12	64	1.552	0.09	17
B-1-c	17.36	82	3.392	0.20	33
B-1-d	20.04	94	1.264	0.08	34
B-1-e	22.28	104	5.520	0.33	36
B-1-f	24.96	115	17.920	1.08	37
B-1-g	31.20	132	4.864	0.29	7
B-1-h	32.88	136	2.528	0.15	28
B-1-i	49.80	172	2.400	0.14	20
B-1-j	55.80	182	2.224	0.13	21
B-1-k	64.28	195	3.216	0.19	22
B-1-l	71.60	204	6.576	0.40	24
B-1-m	78.28	211	17.008	1.02	26
Total			73.456	4.40	
B-2-a	17.80	82	0.320	0.02	33
B-2-b	25.04	116	1.610	0.10	37
B-2-c	30.96	134	0.390	0.02	7
B-2-d	41.20	159	1.170	0.07	30
B-2-e	48.60	173	0.440	0.03	20
B-2-f	75.80	211	5.820	0.35	26
Total			9.750	0.59	

TABLE XV (Continued)

Peak	Time, min	Temp, °C	Area	Percent of Total Area of "B" Fractions	Peak Designation
B-3-a	7.56	47	3.673	0.22	1
B-3-b	17.96	84	5.070	0.31	33
B-3-c	20.40	97	2.503	0.15	34
B-3-d	22.72	107	4.745	0.29	36
B-3-e	25.08	114	10.920	0.66	37
B-3-f	26.32	118	12.675	0.76	38
B-3-g	30.80	132	8.483	0.51	7
B-3-h	41.40	157	33.638	2.03	30
B-3-i	74.24	209	3.608	0.22	14
B-3-j	76.32	211	8.775	0.53	26
Total			94.090	5.68	
B-4-a	7.28	45	1.160	0.07	1
B-4-b	25.88	115	4.080	0.25	37
B-4-c	27.36	120	1.620	0.10	38
B-4-d	31.44	132	1.040	0.06	7
B-4-e	42.36	158	92.600	5.58	30
B-4-f	74.96	209	11.520	0.69	14
B-4-g	77.04	212	5.060	0.30	26
Total			117.080	7.05	
B-5-a	41.56	159	105.345	6.34	30
B-5-b	77.12	210	72.347	4.36	25
Total			117.692	10.70	

TABLE XV (Continued)

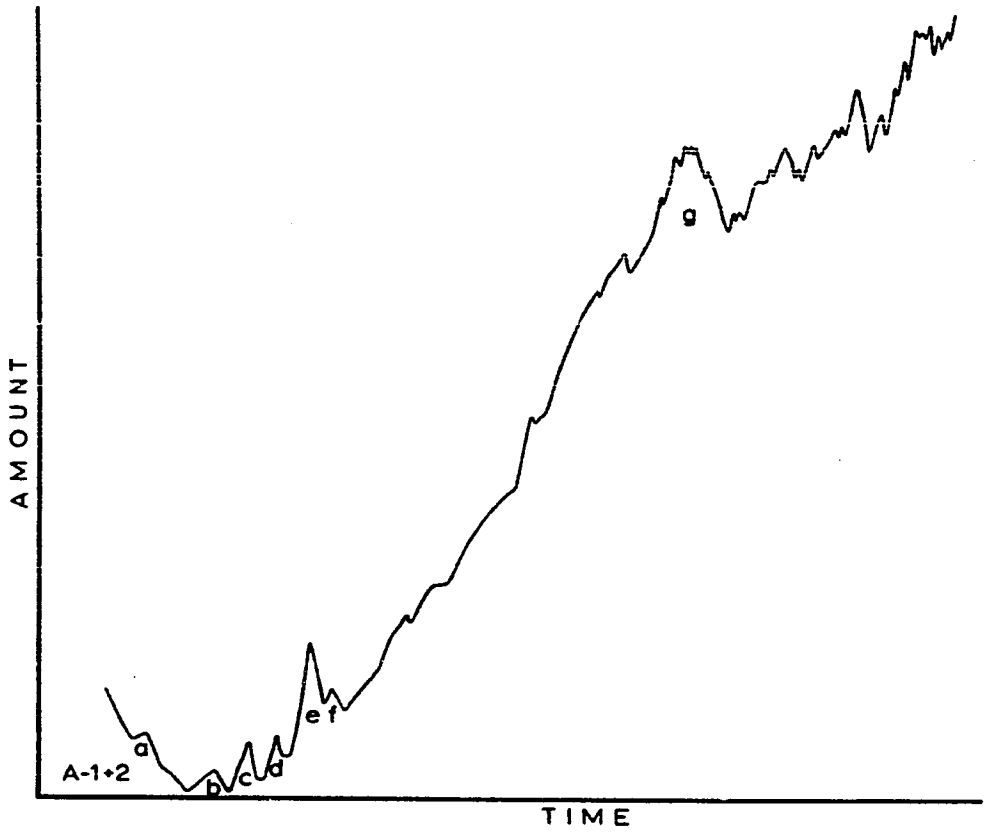
Peak	Time, min	Temp, °C	Area	Percent of Total Area of "B" Fractions	Peak Designation
B-6-a	9.40	57	6.514	0.39	16
B-6-b	23.64	116	6.133	0.37	37
B-6-c	39.16	159	154.399	9.30	30
B-6-d	70.80	210	28.838	1.74	25
B-6-e	73.00	212	6.933	0.42	15
Total			202.817	12.22	
B-7-a	17.96	81	13.491	0.81	3
B-7-b	23.60	106	2.484	0.15	40
B-7-c	31.36	132	2.109	0.13	8
B-7-d	38.44	149	1.358	0.08	29
B-7-e	42.04	158	110.847	6.68	30
B-7-f	74.60	209	44.316	2.67	25
B-7-g	76.80	211	14.011	0.84	15
Total			188.616	11.36	
B-8-a	19.40	86	2.503	0.15	3
B-8-b	31.48	133	1.210	0.07	8
B-8-c	42.64	160	6.518	0.39	30
B-8-d	51.32	177	12.430	0.75	9
B-8-e	60.40	192	11.138	0.67	31
B-8-f	73.64	209	56.980	3.43	32
Total			90.779	5.46	
B-9-a	40.60	162	4.124	0.25	30
B-9-b	49.20	180	10.088	0.61	9
B-9-c	57.60	194	44.704	2.69	31
B-9-d	70.20	213	209.836	12.64	32

TABLE XV (Continued)

Peak	Time, min	Temp, °C	Area	Percent of Total Area of "B" Fractions	Peak Designation
B-10-a	22.28	95	2.903	0.17	35
B-10-b	43.04	161	3.848	0.23	30
B-10-c	60.28	193	55.553	3.35	31
B-10-d	73.20	212	218.768	13.18	32
Total			281.072	16.93	
B-11-a	17.88	84	0.550	0.03	18
B-11-b	30.52	132	1.325	0.08	8
B-11-c	41.20	158	2.675	0.16	30
B-11-d	56.44	186	6.350	0.38	10
B-11-e	59.88	192	34.625	2.09	31
B-11-f	63.44	197	3.275	0.20	23
B-11-g	73.80	210	50.225	3.03	32
B-11-h	75.64	211	36.325	2.19	27
Total			135.350	8.16	
B-12-a	17.72	85	0.896	0.05	18
B-12-b	24.64	116	2.212	0.13	19
B-12-c	30.04	134	1.372	0.08	8
B-12-d	40.72	159	1.512	0.09	30
B-12-e	59.20	193	3.066	0.18	31
B-12-f	72.04	209	5.306	0.32	32
B-12-g	74.88	212	6.216	0.37	27
Total			20.580	1.22	
Total of "B" fractions			1660.034		

Fig. 5. Gas chromatographic separation of the methyl esters of the organic acids of Bryophyllum calycinum on a Carbowax 20 M column.

Fraction	Page
A-1+2	117
A-3	117
A-4	118
A-5	118
A-6	119
A-7	119
A-8	120
A-9	120
A-10	121
A-11	121
A-12	122
B-1	123
B-2	123
B-3	124
B-4	124
B-5	125
B-6	125
B-7	126
B-8	126
B-9	127
B-10	127
B-11	128
B-12	128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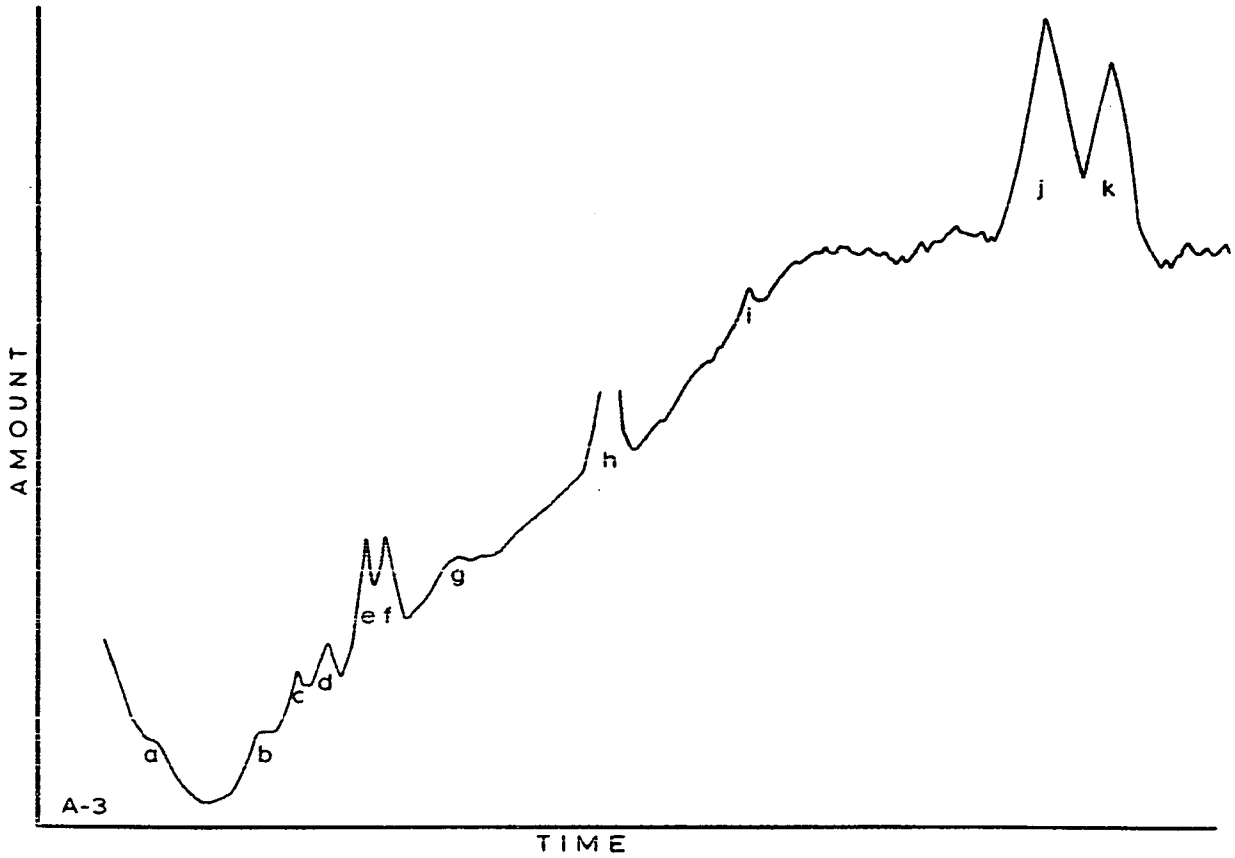
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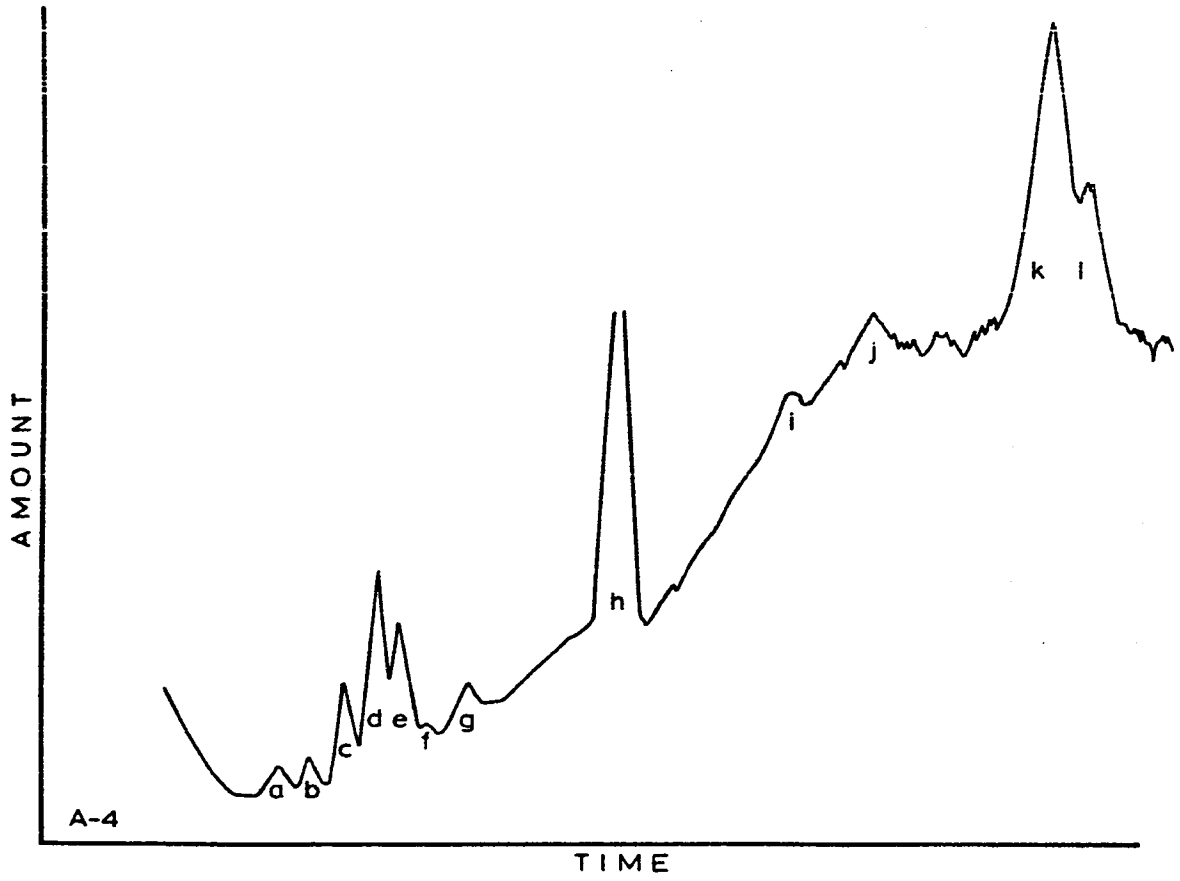
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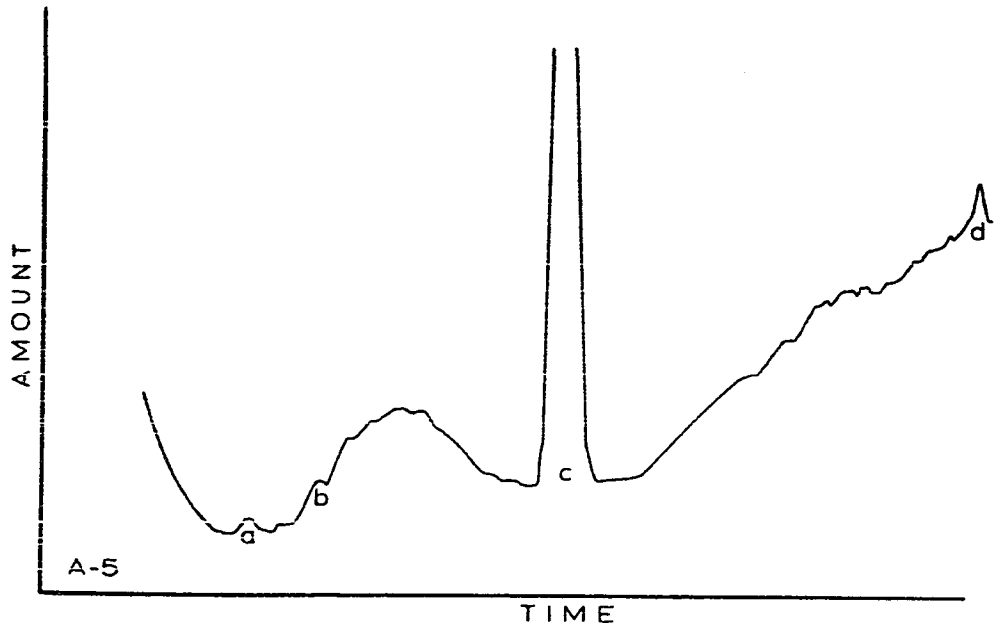
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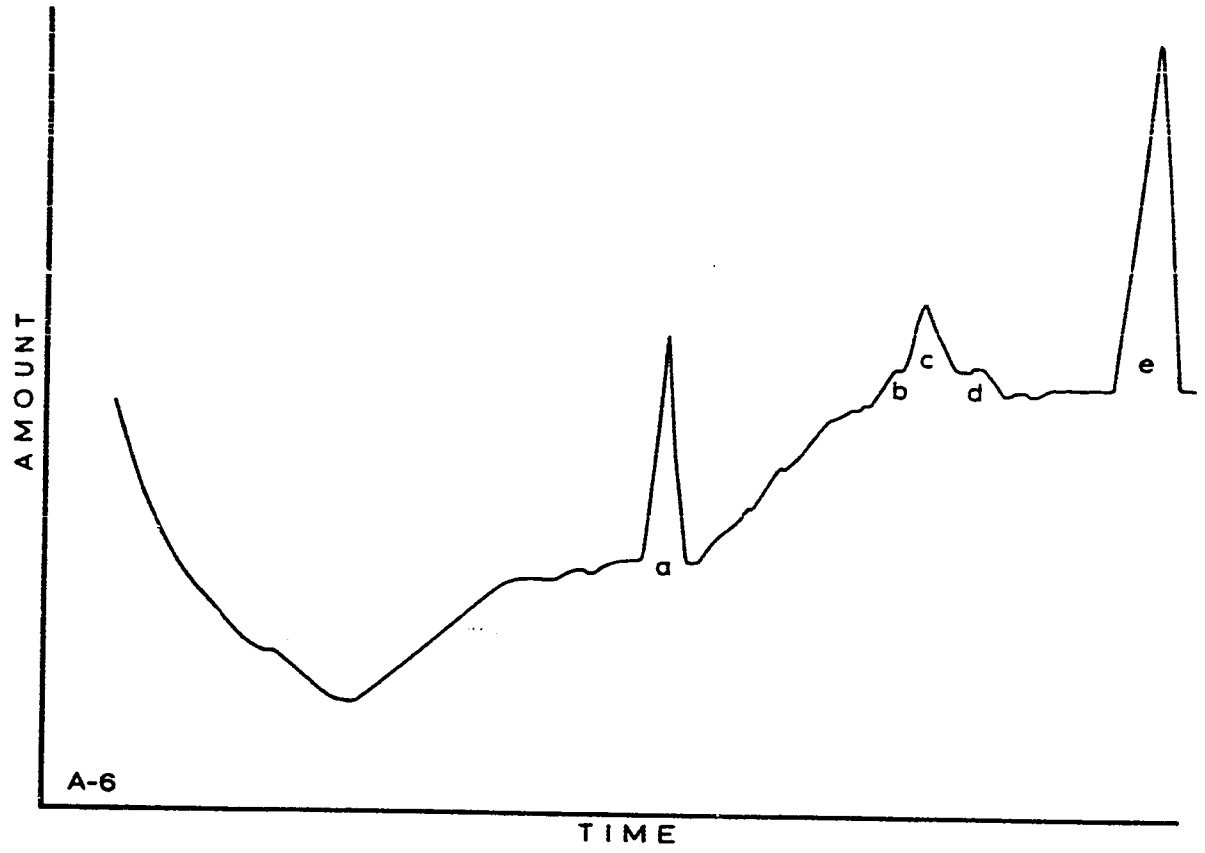
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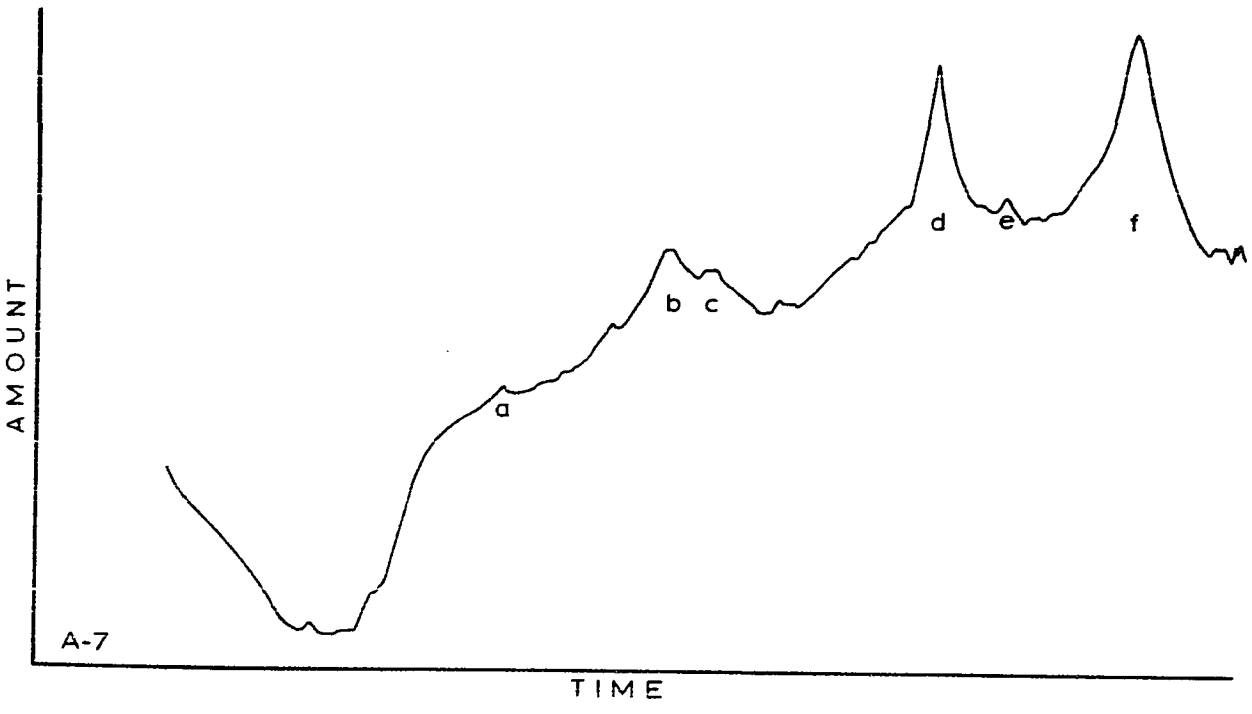
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e
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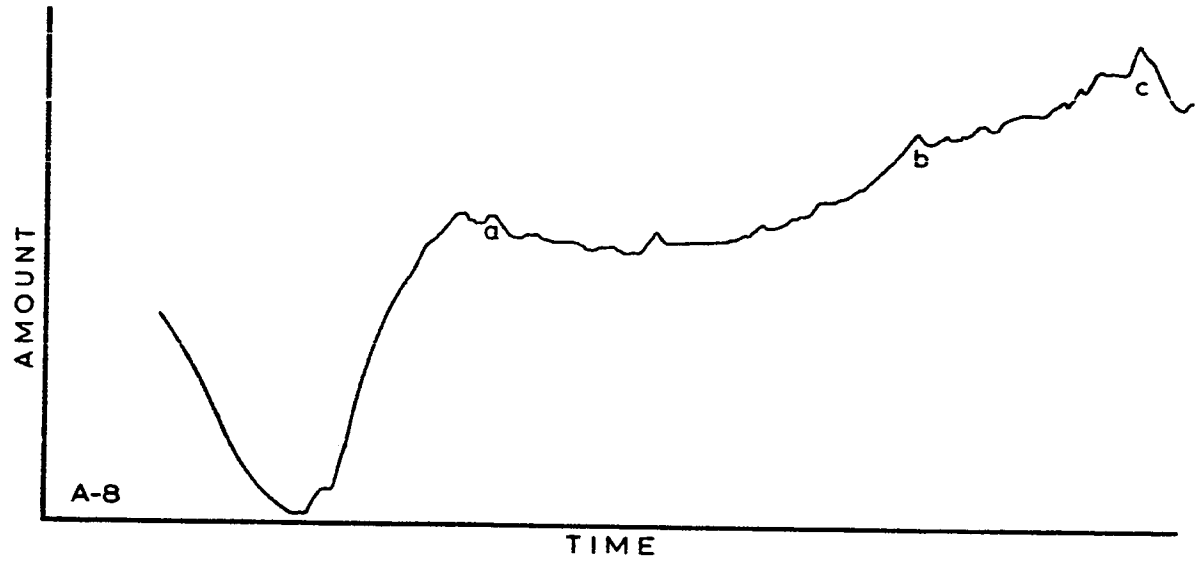
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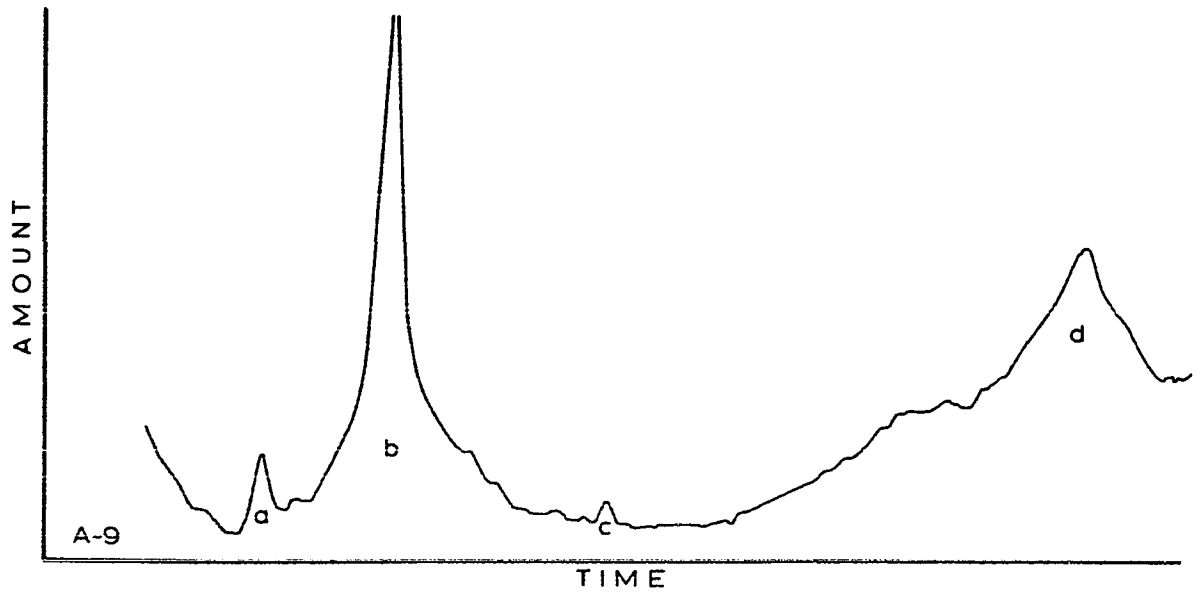
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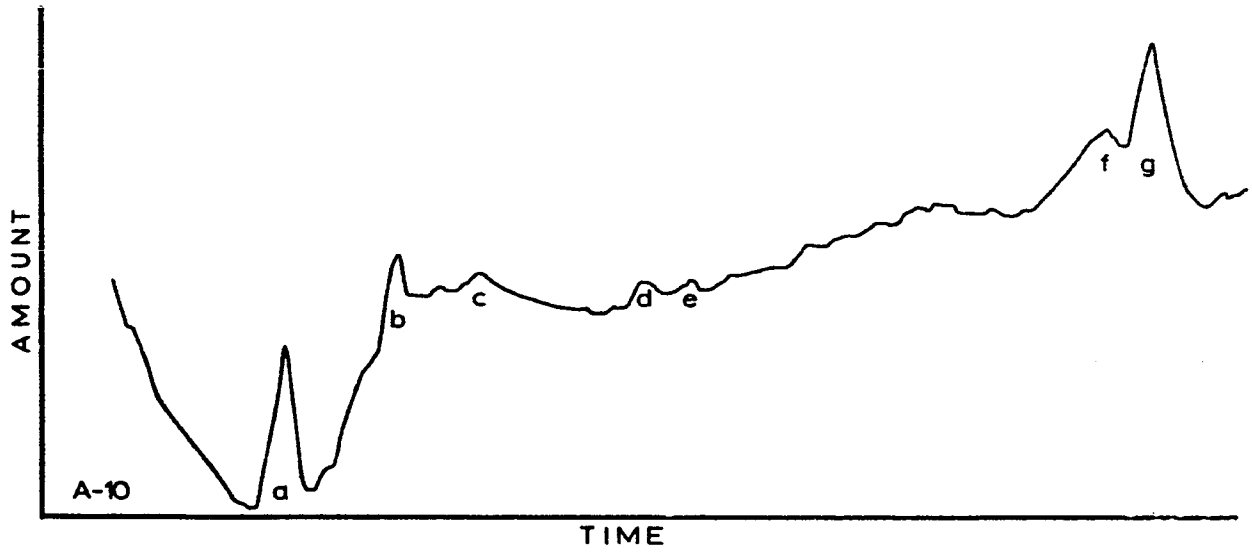
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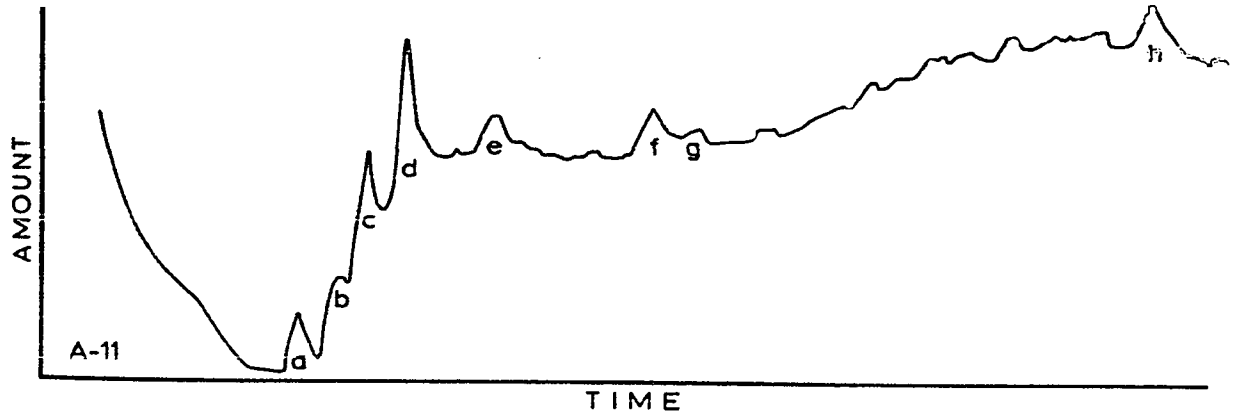
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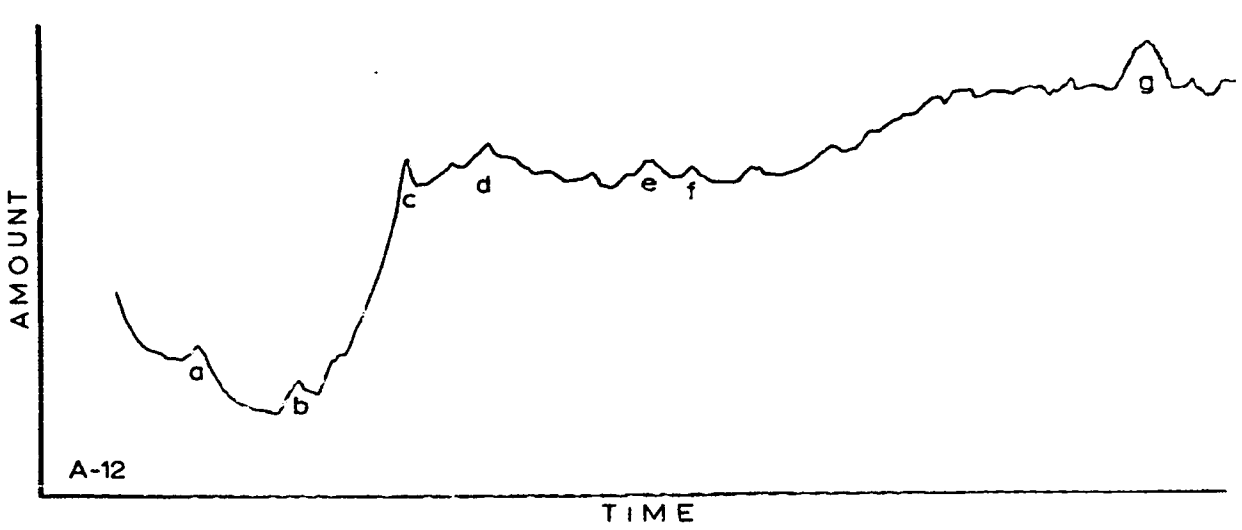
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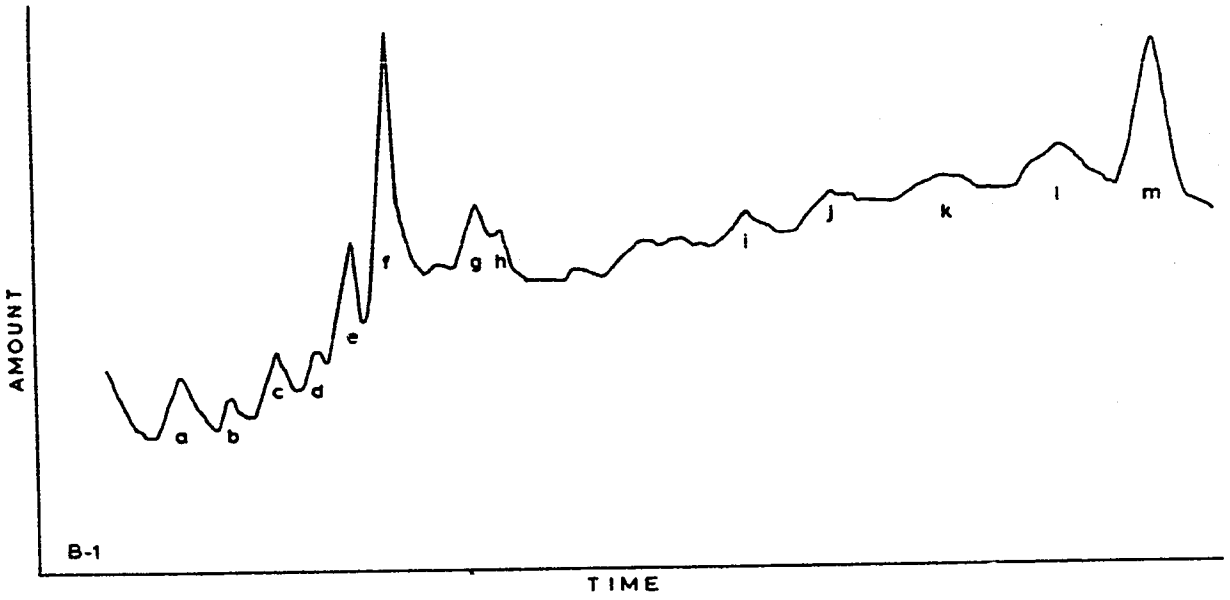
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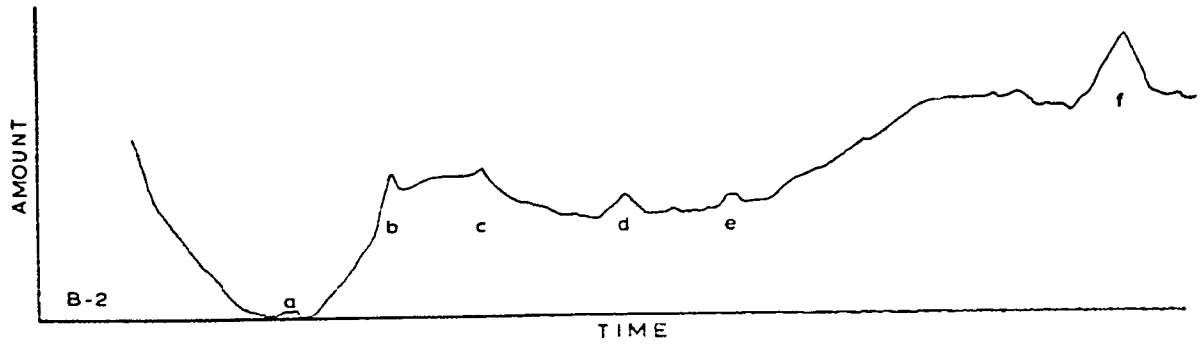
TIME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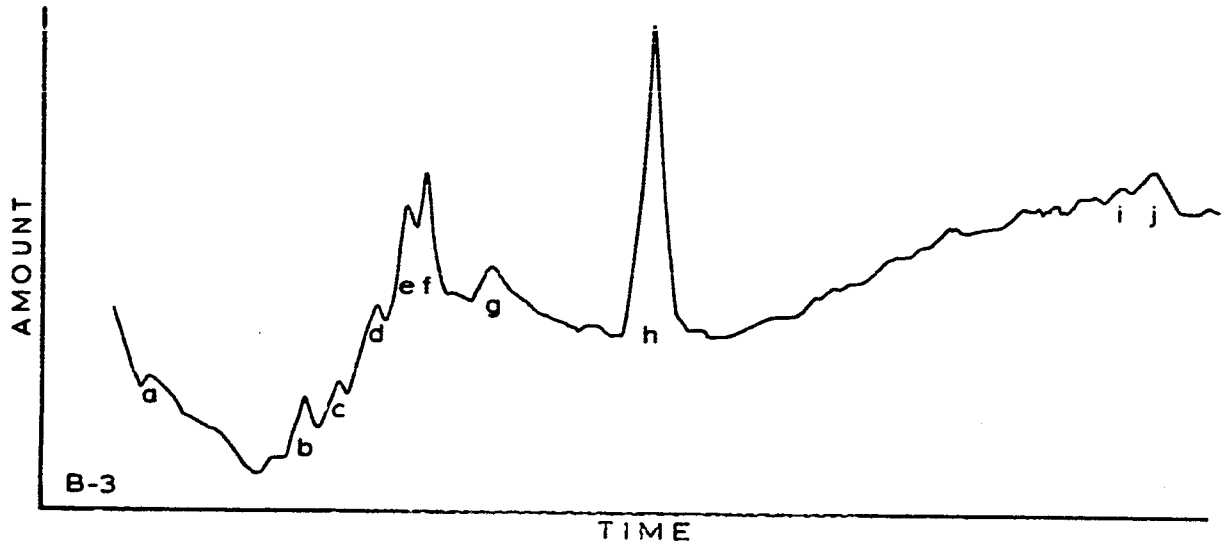
TIME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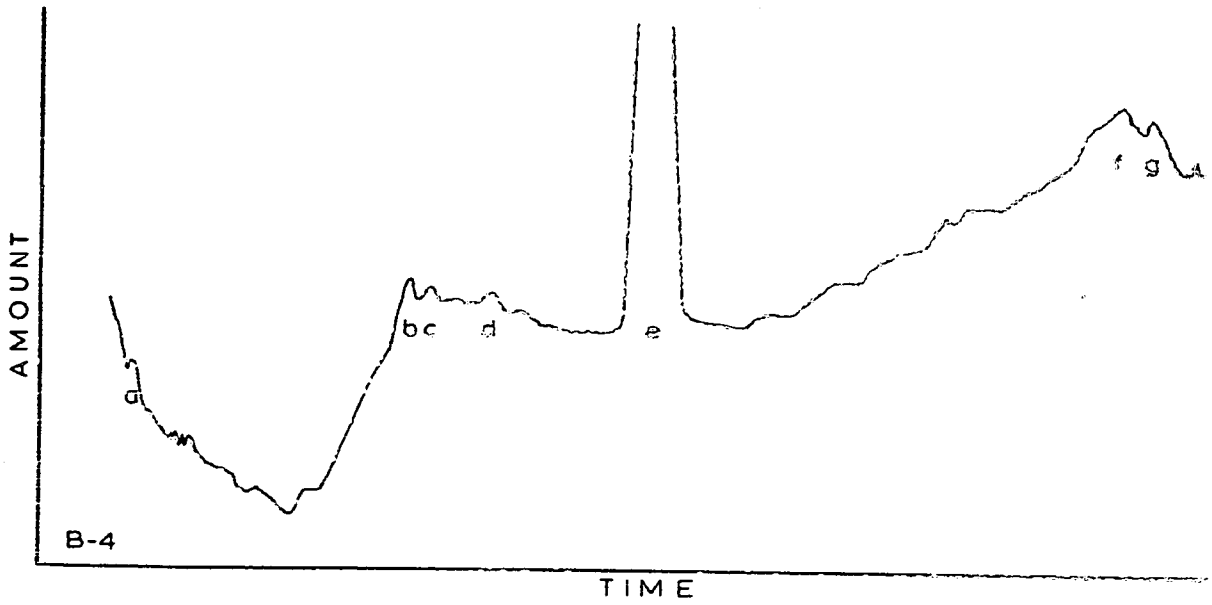
i j
B-3
TIME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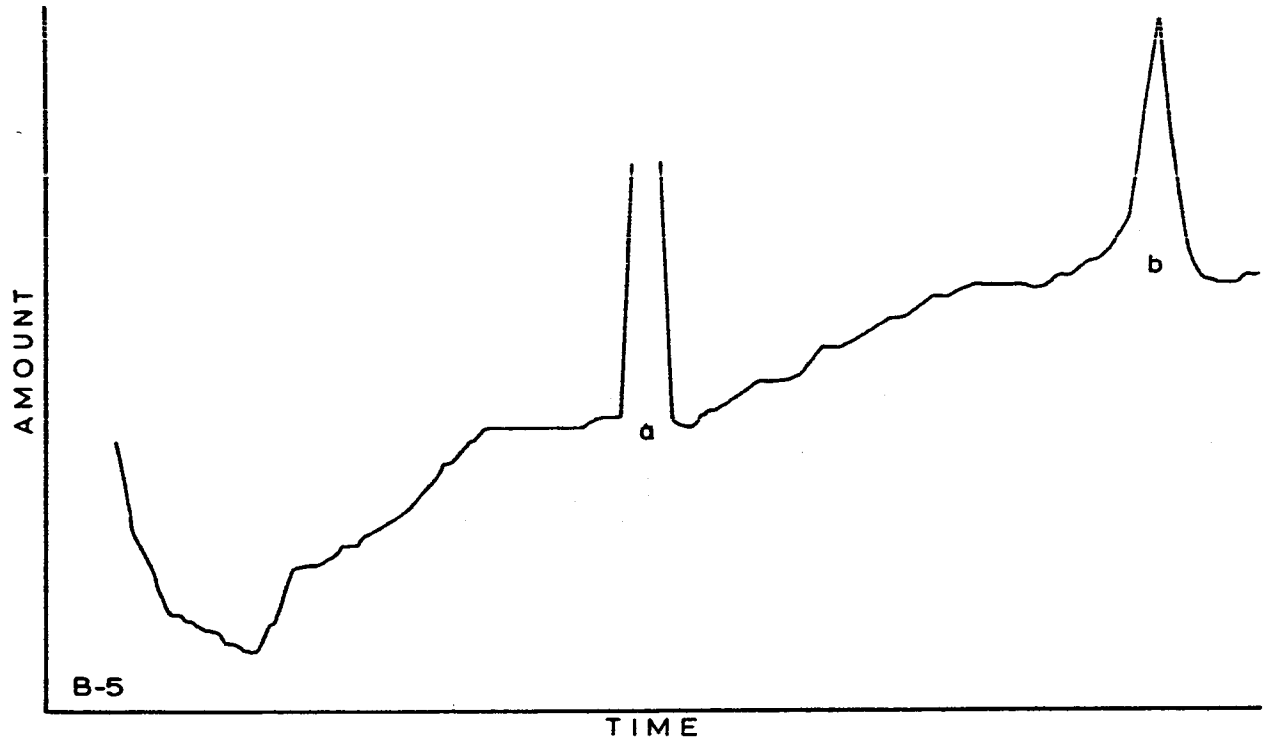
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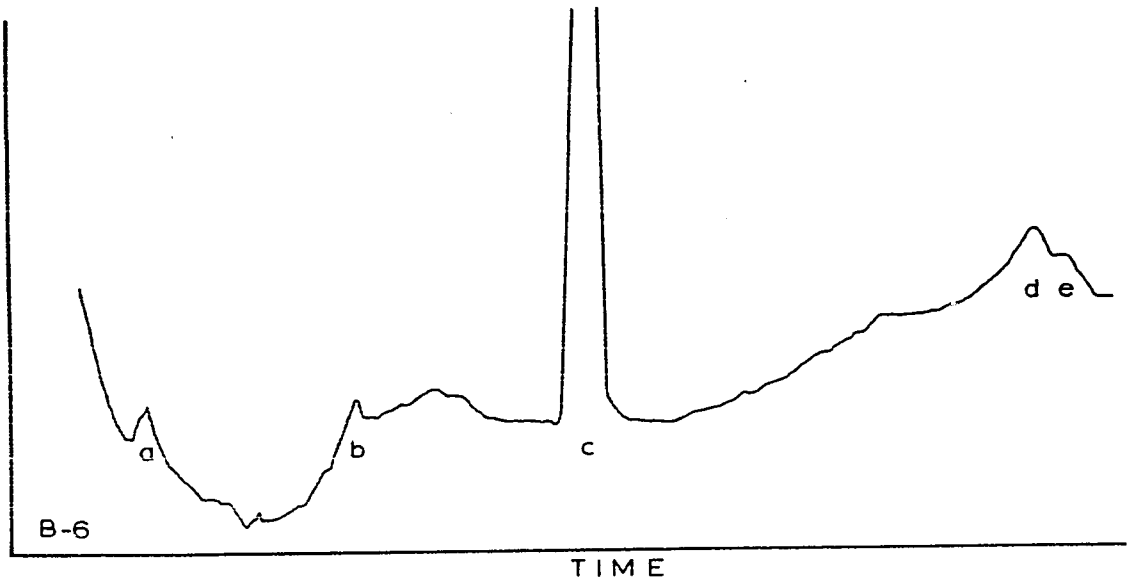
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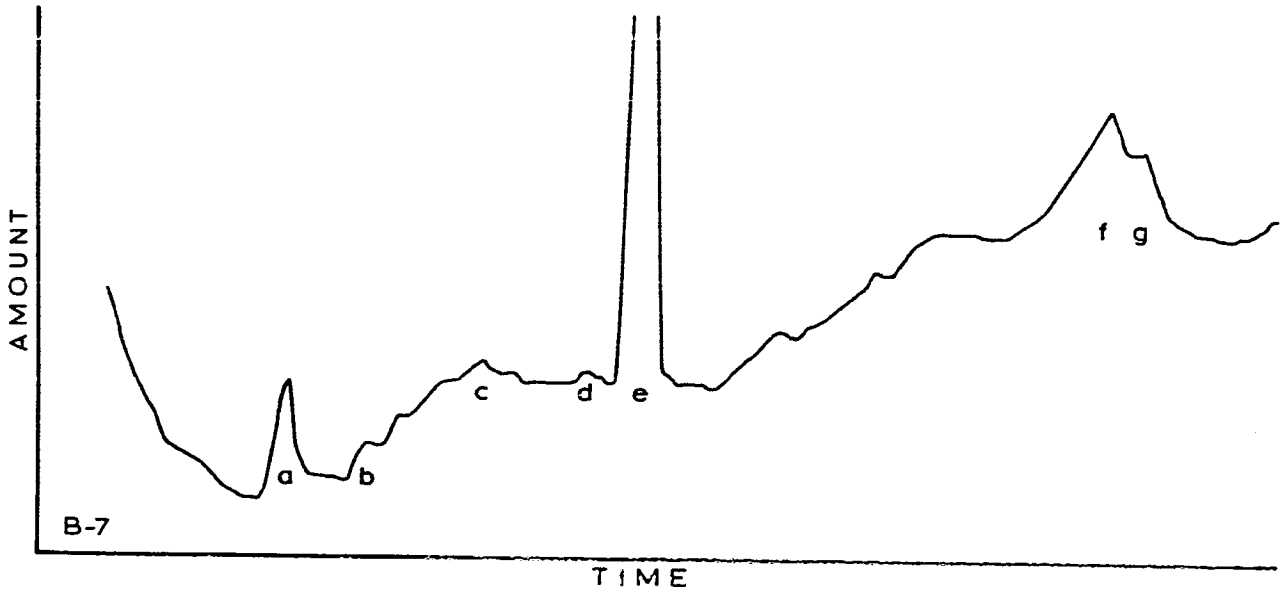
TIME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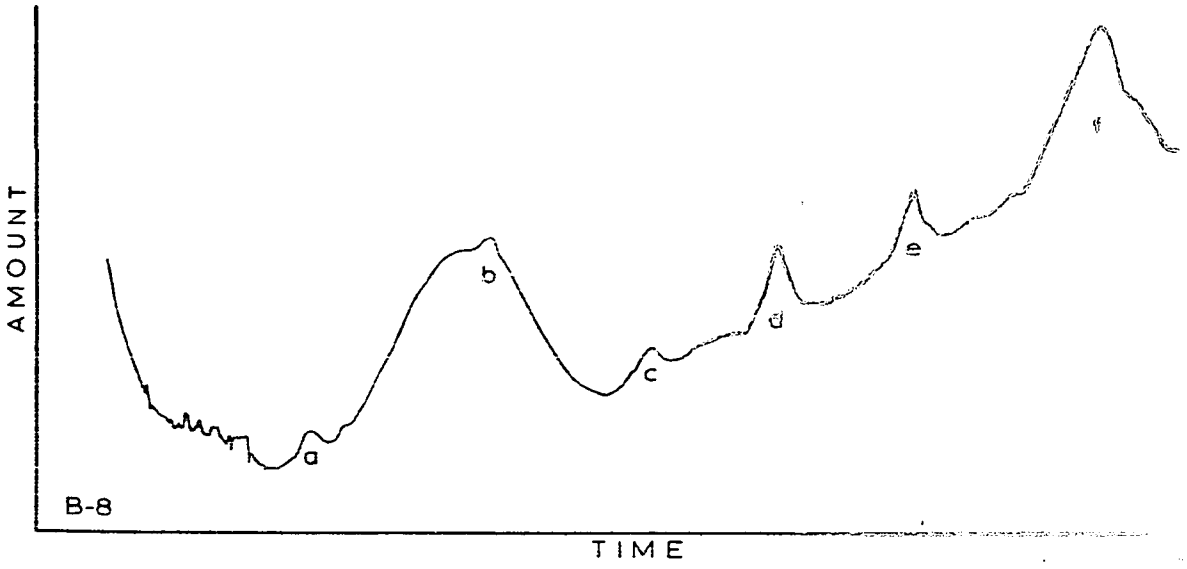
B-7
TIME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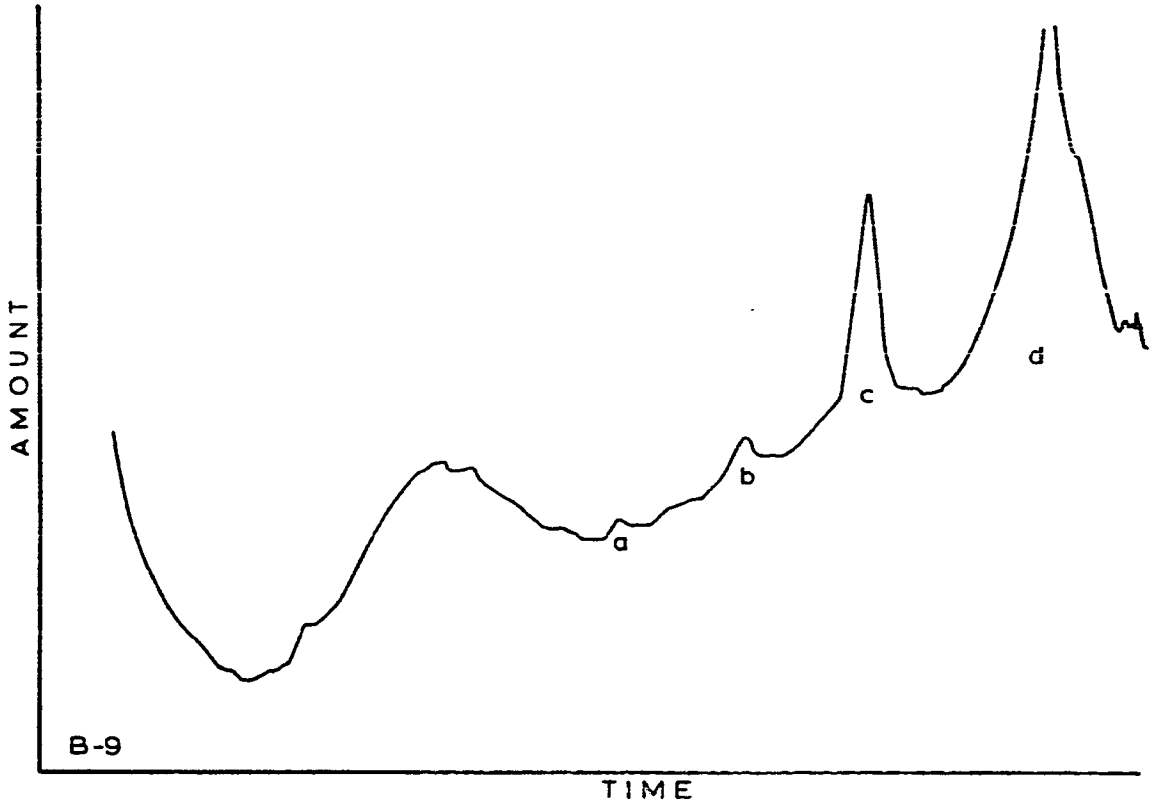
TIME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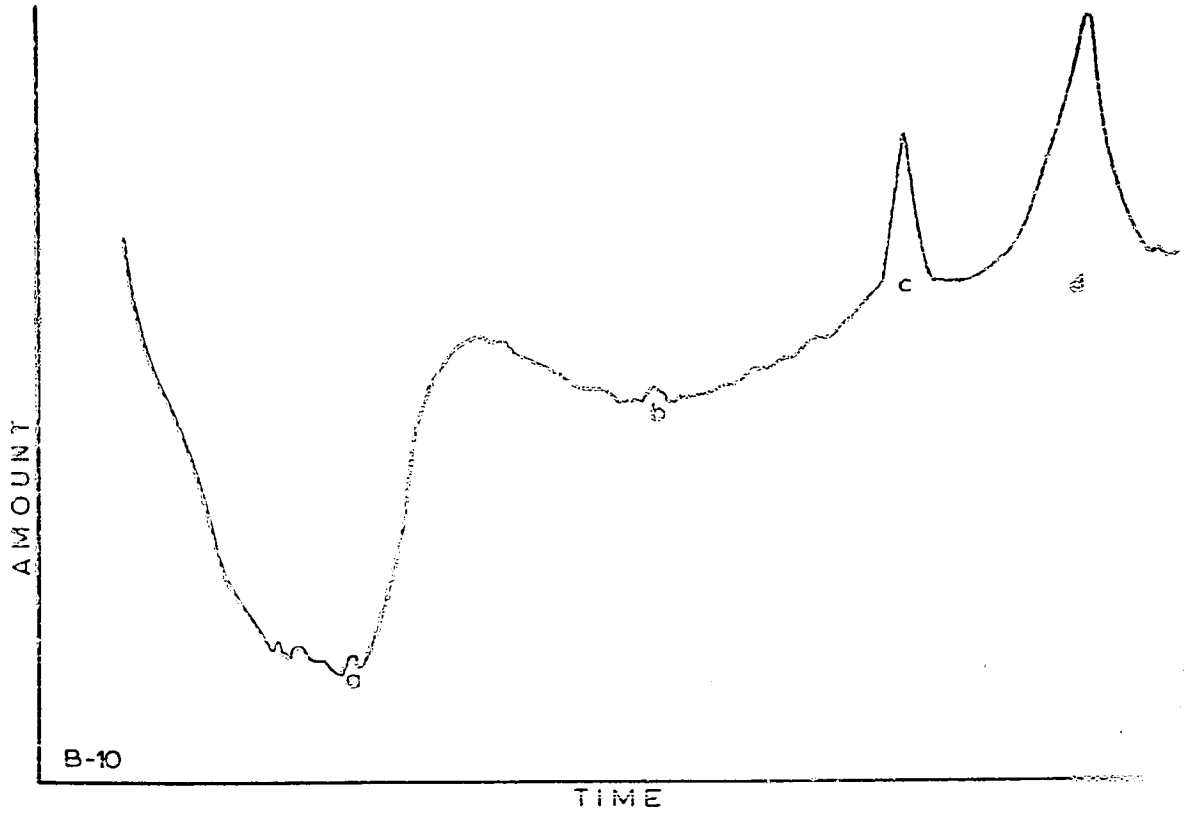
TIME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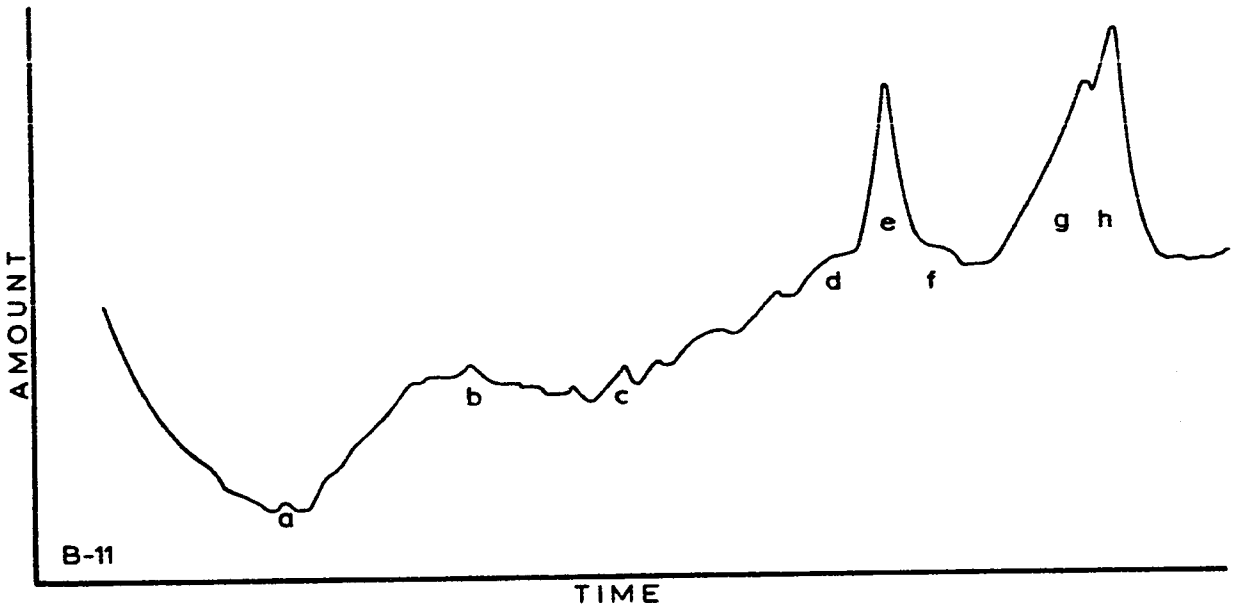
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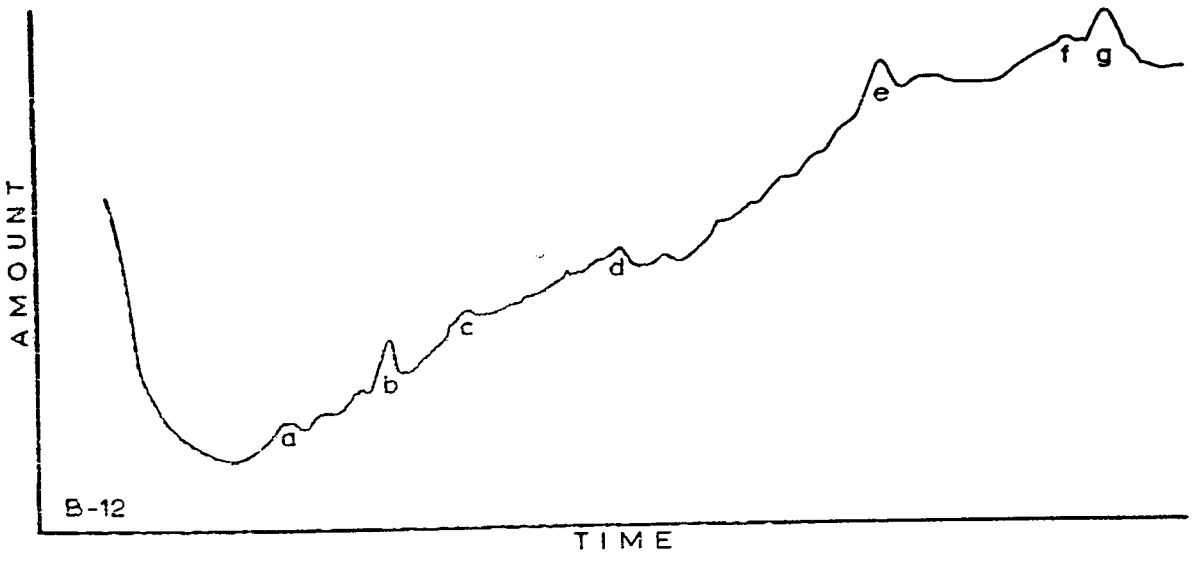
AMOUNT
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B-12
TIME

TABLE XXI

GAS CHROMATOGRAPHIC SEPARATION OF THE METHYL ESTERS OF ORGANIC ACIDS OF BRYOPHYLLUM CALYCINUM

Fraction and Ester	Retention Volume on Carbowax, ml 100°C	150°C	210°C	Elution Temperature from Silicone, °C	Peak Designation
B-2-a	89.8			112	67
B-2-b	205.2				12
B-2-c			61.0		11
B-2-d			91.5	195	10
B-3-a	55.6				7
B-3-b	89.8			110	67
B-3-c		228.4		163	8
B-4-a	51.3				7
B-4-b	89.8			115	67
B-4-c		217.5		162	8
B-5-a	51.3				7
B-5-b	89.8			113	67
B-5-c		228.4			8
B-6-a	51.3				7
B-6-b	89.8			116	67
B-6-c			[illegible]		9
B-7-a	55.6			103	6
B-8-a	51.3			96	68
B-8-b	77.0				2
B-8-c	183.8			136	3

TABLE XXI (Continued)

Fraction and Ester	Retention Volume on Carbowax, ml 100°C	150°C	210°C	Elution Temperature from Silicone, °C	Peak Designation
B-8-d	239.4				4
B-8-e			195.2	196	5
B-9-a	59.9			97	68
B-9-b	94.1			112	1
C-2-a		221.1			8
C-3-a	85.5			111	67
C-3-b		235.6		161	8
C-4-a	85.5			110	67
C-4-b		242.9		161	8
C-5-a	89.8			111	67
C-5-b		192.1			18
C-6-a	85.5			110	67
C-6-b		206.6			18
C-7-a	55.6			103	6
C-7-b	77.0				2
C-7-c		195.8		133	18
C-7-d		195.8		158	19
C-7-e			189.1		5
C-8-a	55.6			96	68
C-8-b	77.0			88	2
C-8-c	77.0			110	15
C-8-d	200.9			120	16
C-8-e	200.9			127	17

TABLE XXI (Continued)

Fraction and Ester	Retention Volume on Carbowax, ml 100°C	150°C	210°C	Elution Temperature from Silicone, °C	Peak Designation
C-8-f	200.9			142	3
C-8-g	252.2				4
C-8-h		145.0		163	13
C-8-i		362.5	51.9	171	14
C-8-j			207.4		5
C-9-a	64.1			98	68
C-9-b	89.8			114	1
C-9-c	196.7				3
C-9-d	252.2				4
D-2-a	55.6				24
D-2-b	89.8			110	67
D-3-a	55.6				24
D-3-b	94.1			112	67
D-4-a	89.8			112	67
D-5-a	51.3				23
D-5-b	85.5				67
D-6-a	85.5				67
D-8-a	51.3				6
D-8-b	89.8				22
D-8-c		210.3			69
D-9-a	89.8				22
D-9-b		210.3			69

TABLE XXI (Continued)

Fraction and Ester	Retention Volume on Carbowax, ml 100°C	150°C	210°C	Elution Temperature from Silicone, °C	Peak Designation
D-10-a	55.6				68
D-10-b	94.1				22
D-10-c		217.5		166	69
D-11-a	55.6			102	68
D-11-b	77.0				2
D-11-c	205.2				3
D-11-d	265.1			150	4
D-11-e		206.6			69
D-11-f			198.3		5
D-12-a	55.6			98	68
D-12-b	94.1			108	20
D-12-c	94.1			114	1
D-12-d	282.2			151	4
D-12-e			91.5		21
E-1-a	64.1				24
E-1-b	98.3			116	32
E-1-c		210.3			69
E-1-d		268.3			36
E-1-e			167.8		35
E-1-f			83.5		34
E-2-a	55.6				24
E-2-c		201.0			36
E-3-a	89.8				67
E-3-b	-	199.4			69

TABLE XXI (Continued)

Fraction and Ester	Retention Volume on Carbowax, ml 100°C	150°C	210°C	Elution Temperature from Silicone, °C	Peak Designation
E-3-c			256.2		33
E-3-d				204	31
E-4-a	59.9				23
E-4-b	94.1			111	67
E-4-c		199.4			69
E-5-a	51.3				23
E-5-b	89.8			108	67
E-5-c		221.1		160	69
E-5-d			112.9		70
E-5-e			234.9	198	71
E-6-a	111.2			118	22
E-6-b		221.1		158	69
E-6-c				206	30
E-7-a		221.1		162	69
E-7-b			109.8		70
E-7-c			228.8	197	29
E-7-d			228.8	202	71
E-8-a	149.6				26
E-8-b	213.8			152	28
E-8-c	265.1				4
E-8-d		210.2		162	69
E-9-a	59.9			100	68
E-9-b	81.2				2
E-9-c	171.0			136	72

TABLE XXI (Continued)

Fraction and Ester	Retention Volume on Carbowax, ml 100°C	150°C	210°C	Elution Temperature from Silicone, °C	Peak Designation
E-9-d	277.9			148	4
E-9-e	483.1	68.9		158	25
E-9-f		210.3		162	69
E-9-g		297.3			66
E-9-h			64.1		27
E-9-i			100.7		21
F-1-a	59.9			99	24
F-1-b	98.3				67
F-1-c			253.2	199	45
F-2-a	59.9			102	24
F-2-b	94.1			116	67
F-2-c	256.5				47
F-2-d		184.9		148	48
F-2-e		290.0			73
F-2-f			210.5		50
F-3-a	55.6				23
F-3-b	94.1			111	67
F-3-c			237.9		71
F-4-a	55.6				23
F-4-b	94.1			115	67
F-4-c				150	57
F-4-d		219.3			69
F-4-e				171	40
F-4-f				181	49

TABLE XXI (Continued)

Fraction and Ester	Retention Volume on Carbowax, ml 100°C	150°C	210°C	Elution Temperature from Silicone, °C	Peak Designation
F-4-g			122.0		70
F-4-h			228.8	203	71
F-5-a	145.4			110	42
F-5-b	145.4			138	41
F-5-c		97.9		157	43
F-5-d		97.9		168	44
F-5-e			125.1	198	70
F-5-f			241.0	202	71
F-5-g			286.7	220	46
F-6-a		228.4	39.7	164	69
F-6-b				181	49
F-6-c			115.9	202	70
F-6-d			241.6	206	71
F-7-a	55.6				39
F-7-b		235.6		162	69
F-7-c			112.9	198	70
F-7-d			244.0	202	71
F-8-a	59.9			108	39
F-8-b	205.2				28
F-8-c	260.8				4
F-8-d		235.6		165	69
F-8-e			234.9	202	71
F-9-a	51.3			103	68
F-9-b	77.0				2

TABLE XXI (Continued)

Fraction and Ester	Retention Volume on Carbowax, ml 100°C	150°C	210°C	Elution Temperature from Silicone, °C	Peak Designation
F-9-c	102.6				38
F-9-d	282.2				4
F-9-e			183.0		37
G-1-a	59.9				24
G-1-b	94.1				64
G-1-c			250.1		45
G-2-a	59.9			109	24
G-2-b	85.5				64
G-2-c	303.5				65
G-3-a	132.5				41
G-3-b		83.4			44
G-3-c			152.5		61
G-3-d			234.9	201	71
G-4-a			106.8	198	70
G-4-b			237.9	199	71
G-5-a	55.6			101	68
G-5-b	89.8			117	51
G-5-c	243.7			133	58
G-5-d			106.8	198	70
G-5-e			241.0	199	71
G-6-a	59.9			101	68
G-6-b	81.2			115	51
G-6-c	158.2				59
G-6-d	213.8				53
G-6-e	269.3			148	52

TABLE XXI (Continued)

Fraction and Ester	Retention Volume on Carbowax, ml 100°C	150°C	210°C	Temperature from Silicone, °C	Peak Designation
G-6-f			164.7		37
G-6-g			231.6	202	71
G-7-a	55.6			100	68
G-7-b	81.2			114	51
G-7-c	218.0				53
G-7-d	265.1			148	52
G-7-e			170.8		37
H-1-a	55.6				24
H-1-b	94.1				64
H-2-a	55.6			109	24
H-2-b	94.1				64
H-3-a	59.9				24
H-3-b	316.4				65
H-4-a	55.6				62
H-4-b	94.1				63
H-5-a	59.9				62
H-5-b	94.1				63
H-5-c			250.1	201	71
H-6-a	55.6				68
H-6-b			250.1	201	71
H-7-a	55.6			101	68
H-7-b		210.3		161	69
H-7-c			250.1	199	71
H-8-a	55.6			101	68
H-8-b	89.8			115	51

TABLE XXI (Continued)

Fraction and Ester	Retention Volume on Carbowax, ml			Temperature from Silicone, °C	Peak Designation
	100°C	150°C	210°C		
H-8-c	158.2				54
H-8-d	273.6			148	52
H-8-e		206.6		162	69
H-8-f			256.2	202	71
H-9-a	55.6			100	68
H-9-b	94.1			114	51
H-9-c	171.0			131	54
H-9-d	205.2				53
H-9-e	273.6			148	52
H-9-f		83.4			60
H-9-g		206.6		163	69
H-9-h		500.3	67.1	193	56
H-10-a	59.9			100	68
H-10-b	94.1			114	51
H-10-c	171.0			131	54
H-10-d	269.3			148	52
H-10-e	414.7				55
H-10-f		213.9		163	69
H-10-g		514.8	64.1	193	56

Fig. 9. Infrared spectra of methyl esters of known acids and organic acids of Bryophyllum calycinum.

Methyl ester of known acid	Page *
glyoxylic	142 t
glycolic	142 uc
pyruvic	142 lc
lactic	142 b
oxalic	143 t
malonic	143 uc
succinic	143 lc
fumaric	143 b
malic	144 t
tartaric	144 uc
oxalacetic (peak 1)	144 lc
oxalacetic (peak 2)	144 b
oxalacetic (peak 3)	145 t
α-ketoglutaric (peak 1)	145 uc
α-ketoglutaric (peak 2)	145 lc
cis-aconitic	145 b
citric	146 t
isocitric	146 uc

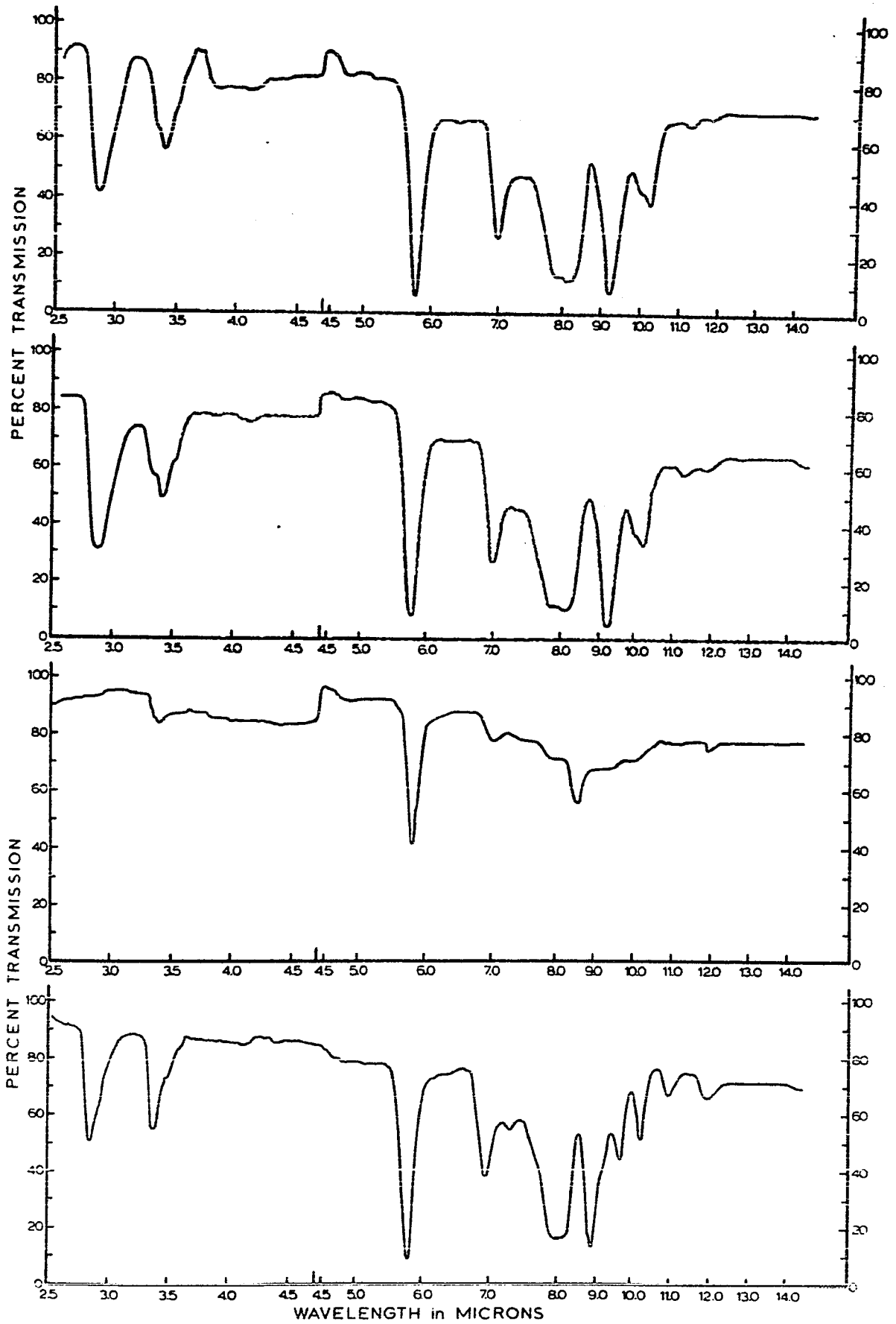
* t : top, uc : upper centre, lc : lower centre, b: bottom

Methyl esters of

organic acids of Bryophyllum calycinum

Fraction and ester	Peak number	Page
D-12-c	1	146 lc
C- 8-b	2	146 b
C- 8-f	3	147 t
E- 9-d	4	147 uc
B- 8-e	5	147 lc
B- 7-a	6	147 b
C- 3-b	8	148 t
B- 2-d	10	148 uc
C- 8-h	13	148 lc
C- 8-i	14	148 b
C- 8-c	15	149 t
C- 8-d	16	149 uc
C- 8-e	17	149 lc
C- 7-c	18	149 b
C- 7-d	19	150 t
D-12-b	20	150 uc
E- 6-a	22	150 lc
F- 2-a	24	150 b
E- 9-e	25	151 t
E- 8-b	28	151 uc
E- 7-c	29	151 lc
E- 6-c	30	151 b
E- 3-d	31	152 t
E- 1-b	32	152 uc

Fraction and ester	Peak number	Page
F- 8-a	39	152 lc
F- 4-e	40	152 b
F- 5-b	41	153 t
F- 5-a	42	153 uc
F- 5-c	43	153 lc
F- 5-d	44	153 b
F- 1-c	45	154 t
F- 5-g	46	154 uc
F- 2-d	48	154 lc
F- 6-b	49	154 b
G- 7-b + H- 9-b + H-10-b	51	155 t
G- 7-d + H- 9-e + H-10-d	52	155 uc
H- 9-c + H-10-c	54	155 lc
H- 9-h + H-10-g	56	155 b
F- 4-c	57	156 t
G- 5-c	58	156 uc
E- 5-b	67	156 lc
D-12-a	68	156 b
E- 6-b	69	157 t
F- 5-e	70	157 uc
F- 6-d	71	157 lc
E- 9-c	72	157 b


PERCENT TRANSMISSION
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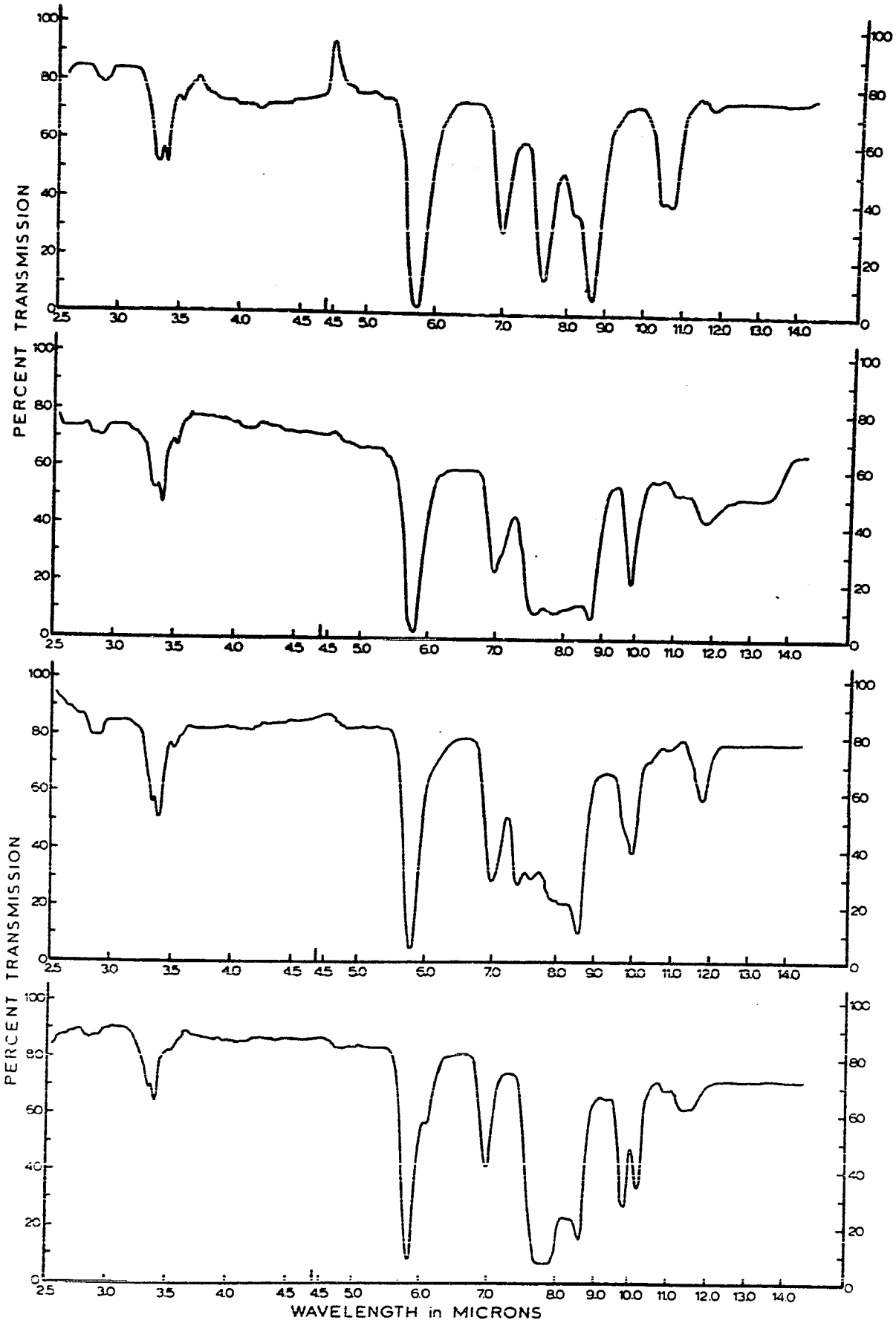
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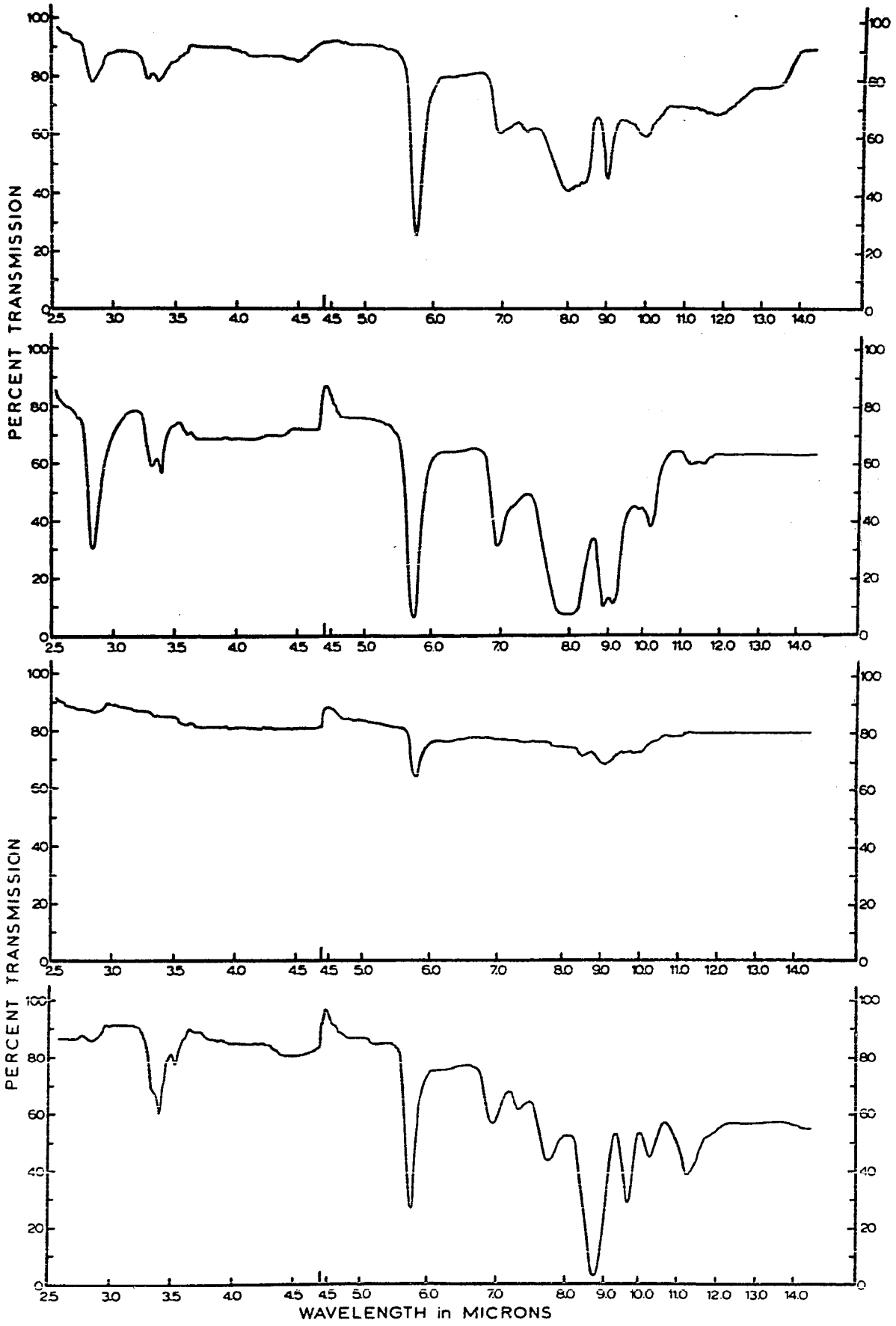
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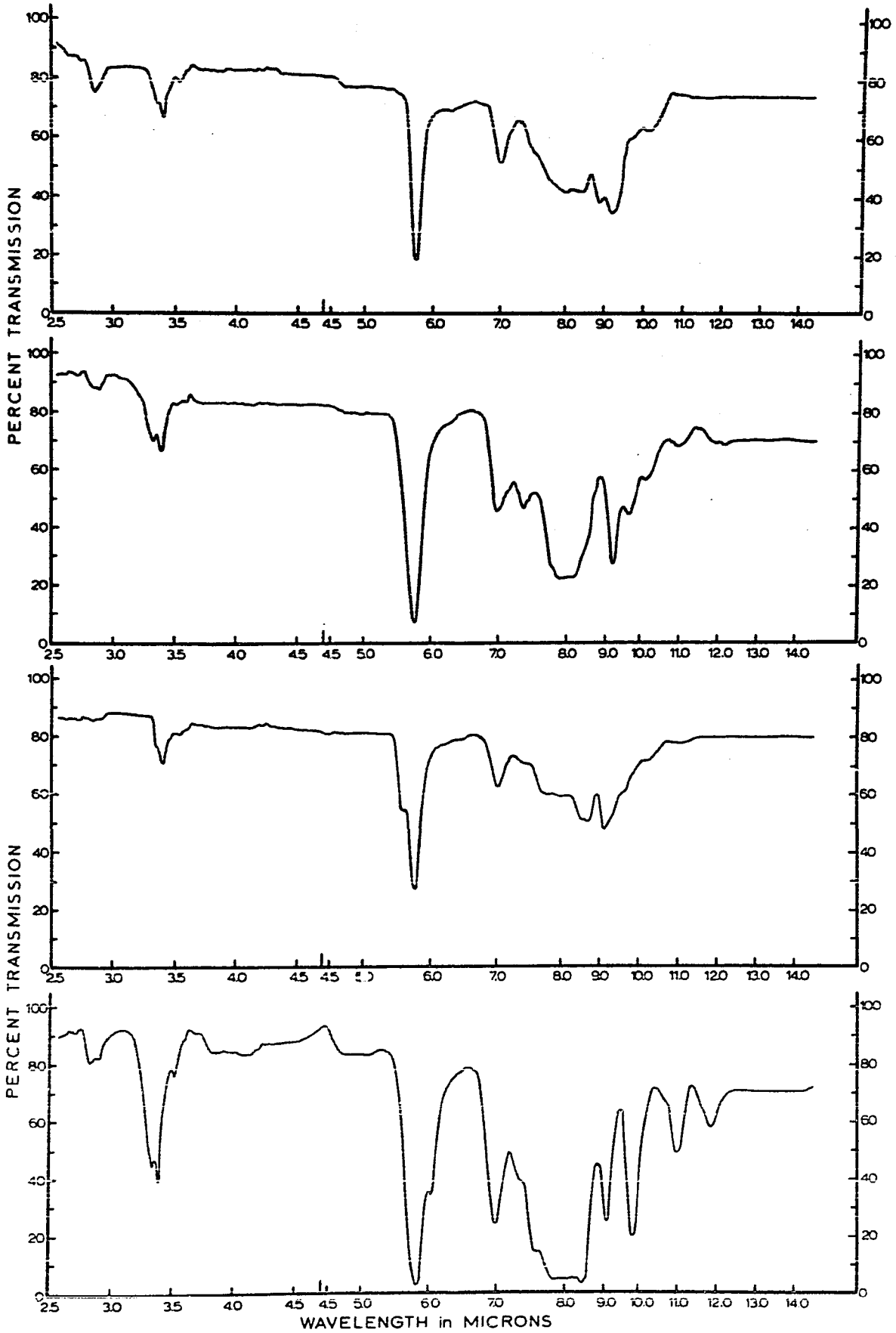
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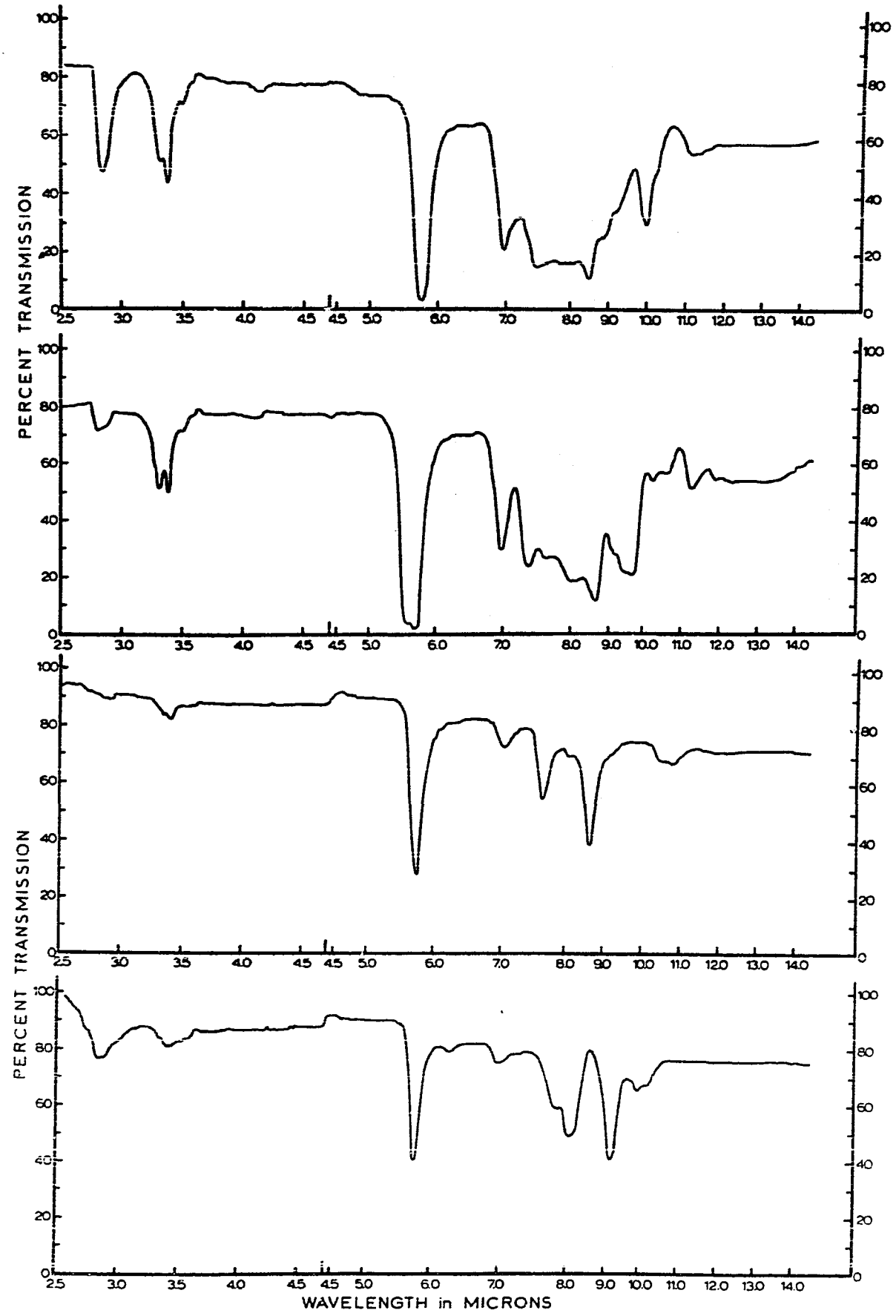
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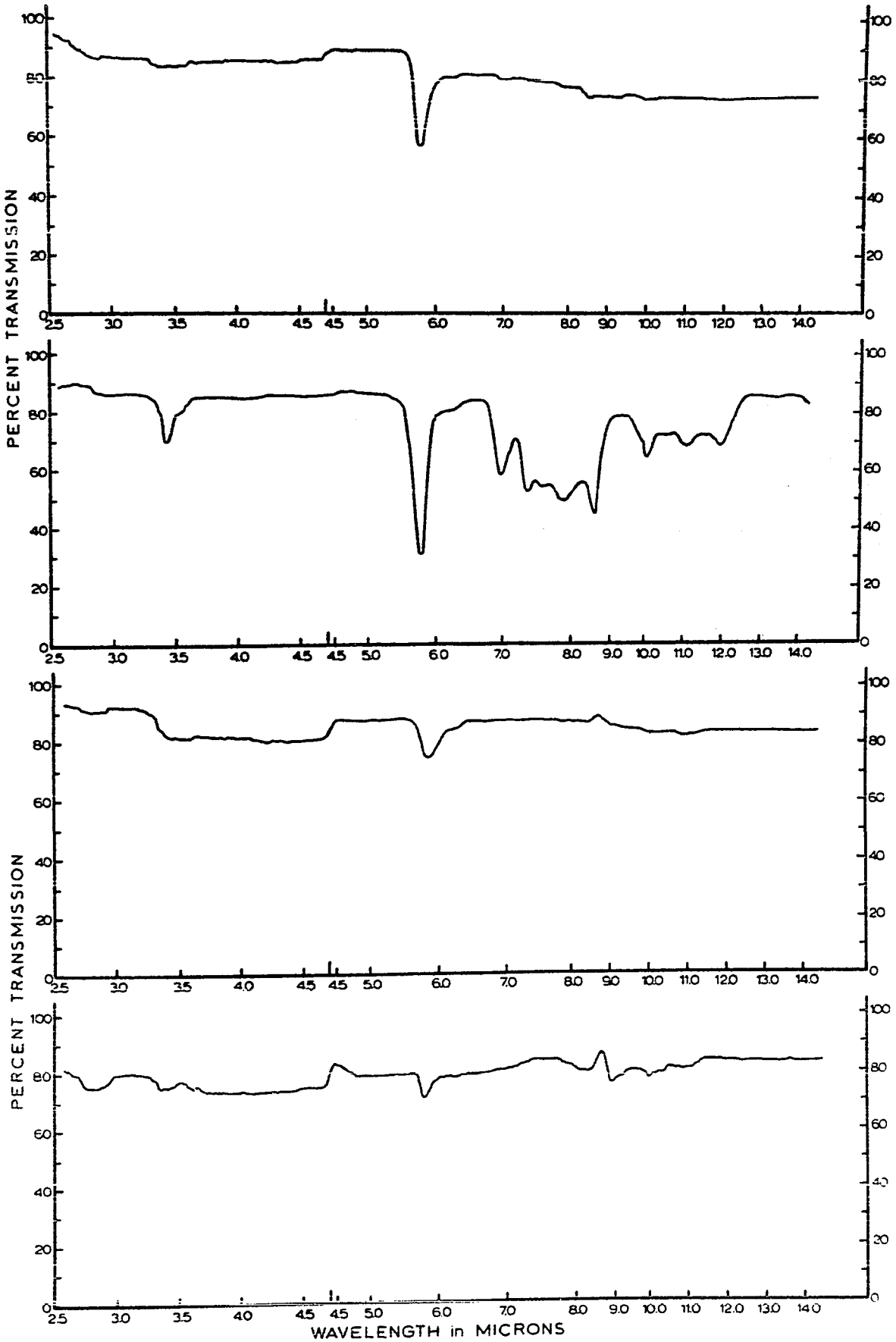
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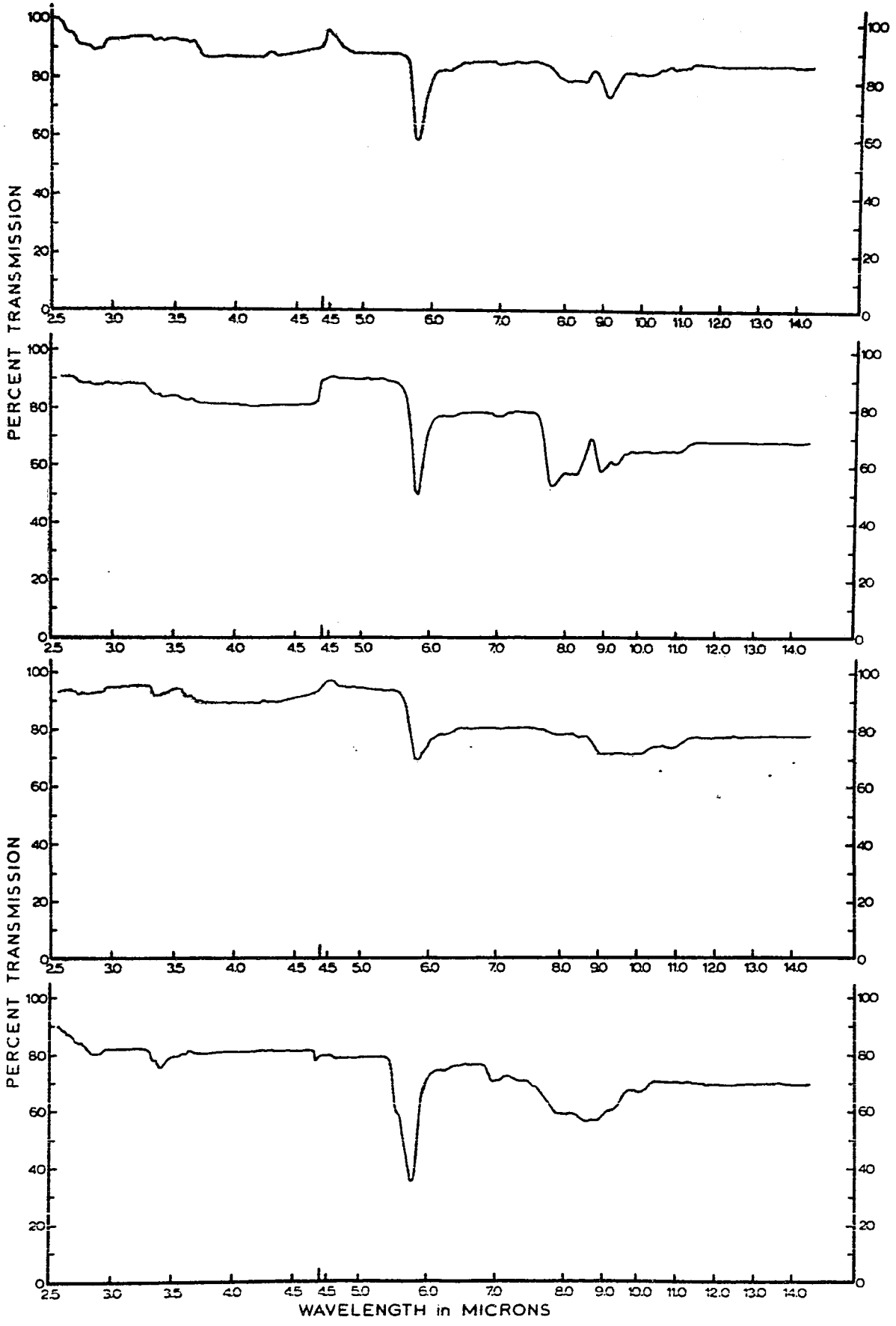
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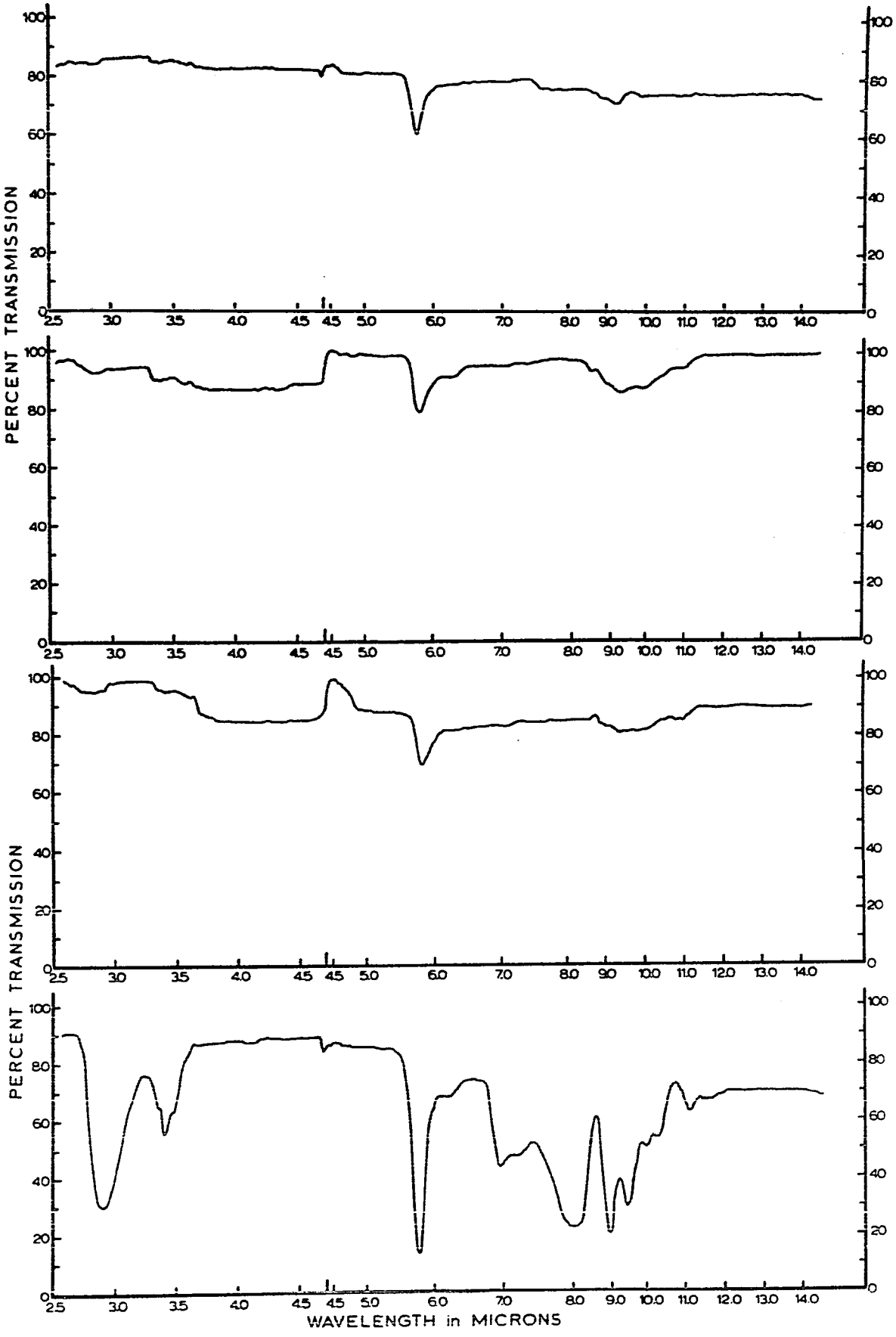
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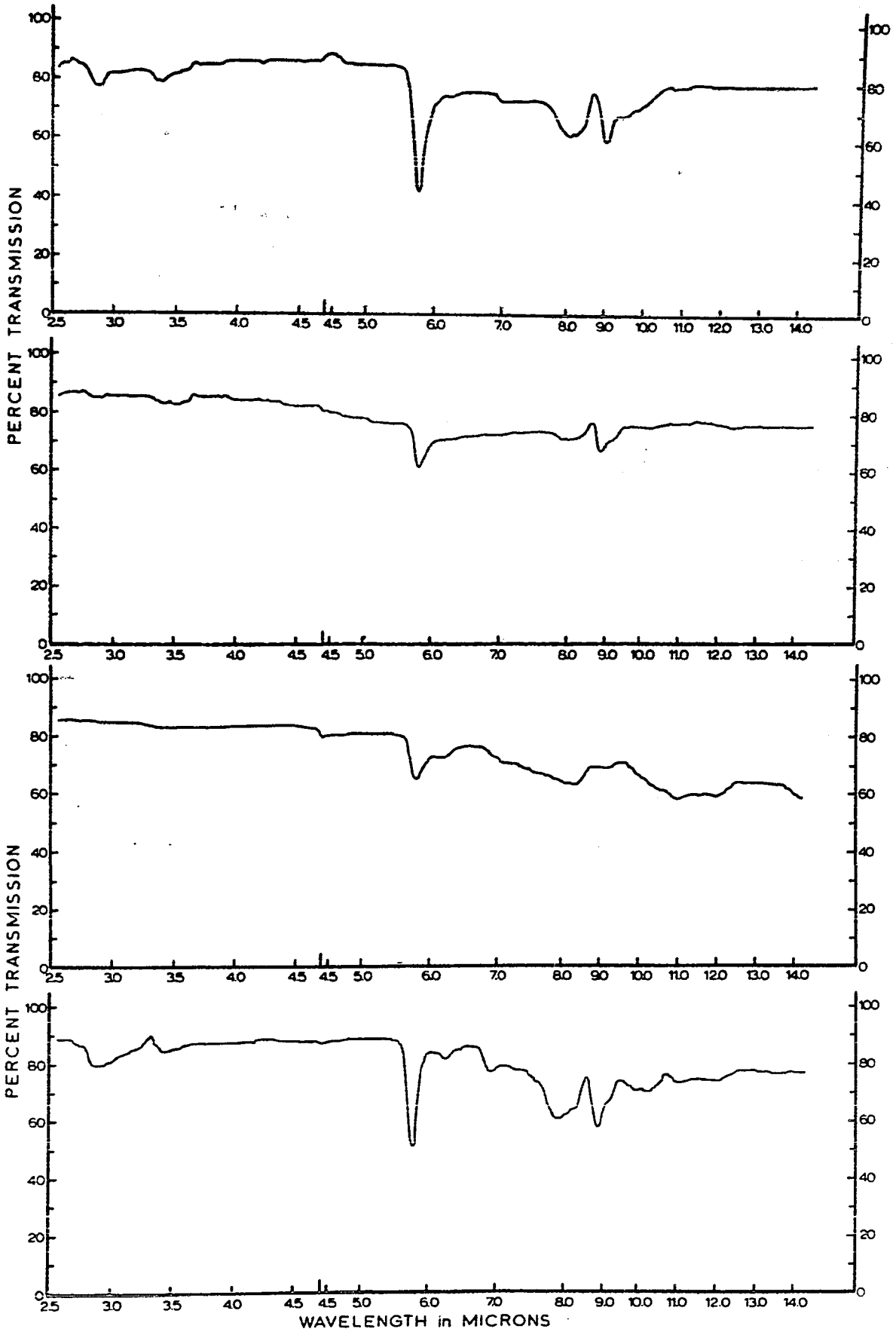
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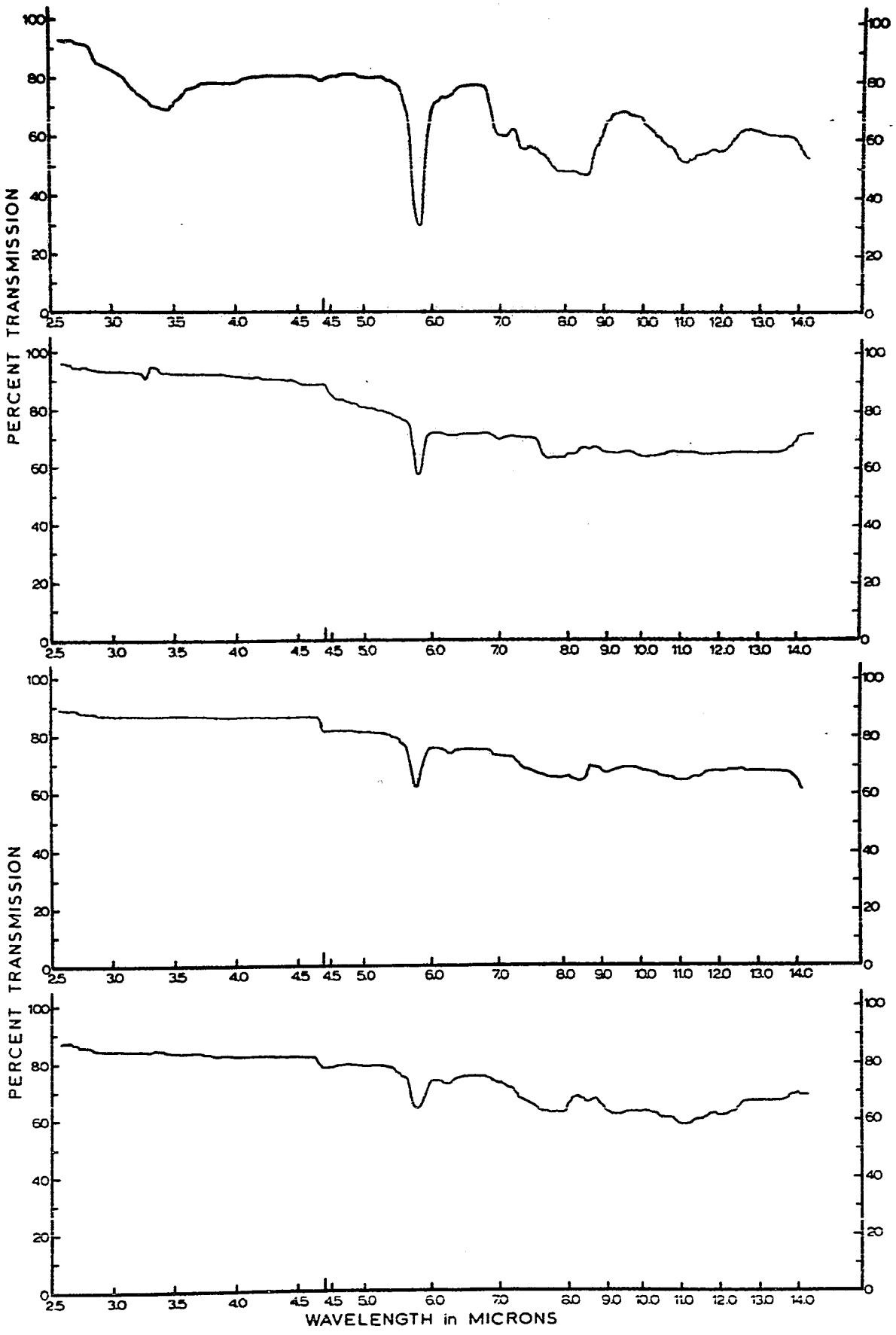
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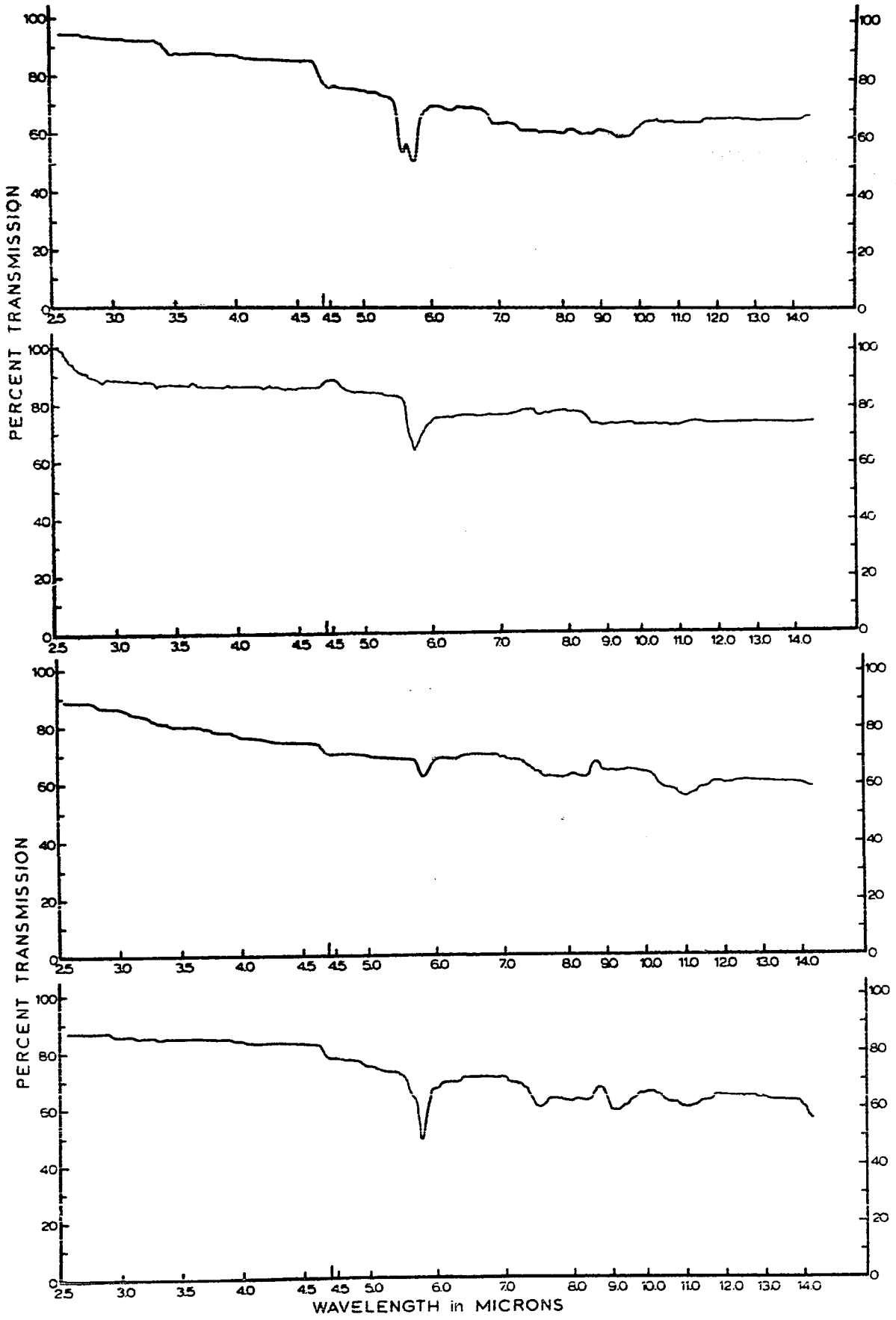
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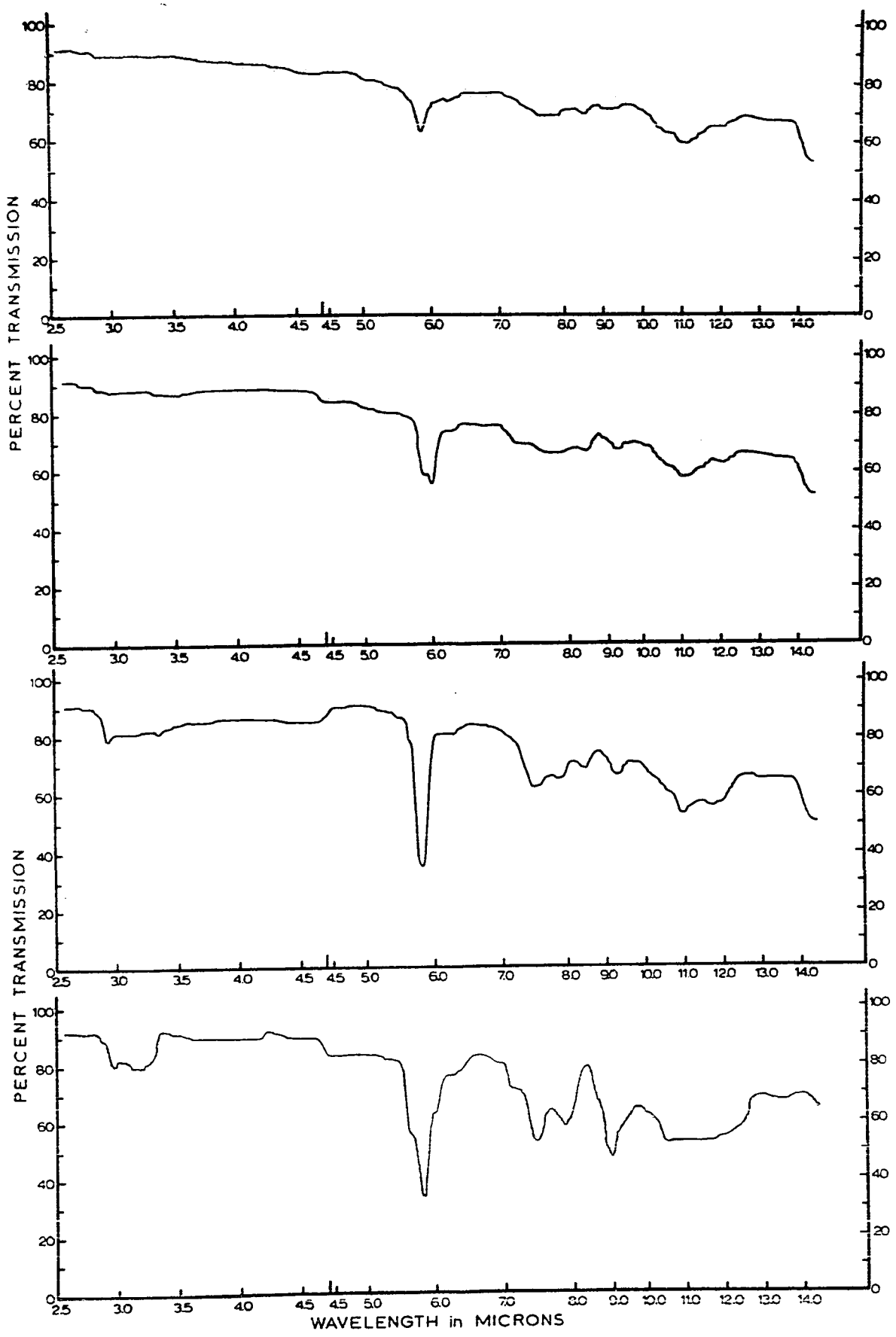
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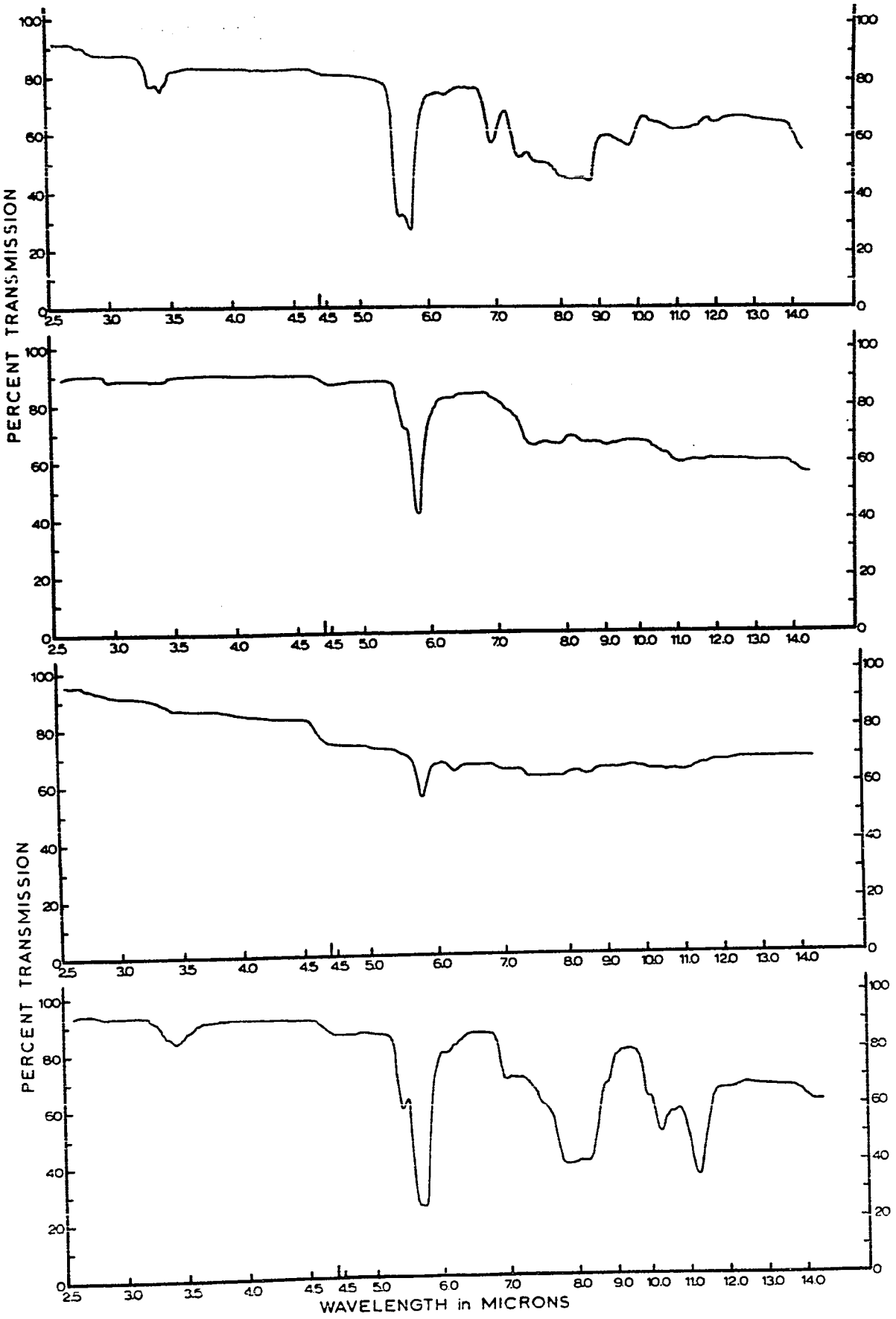
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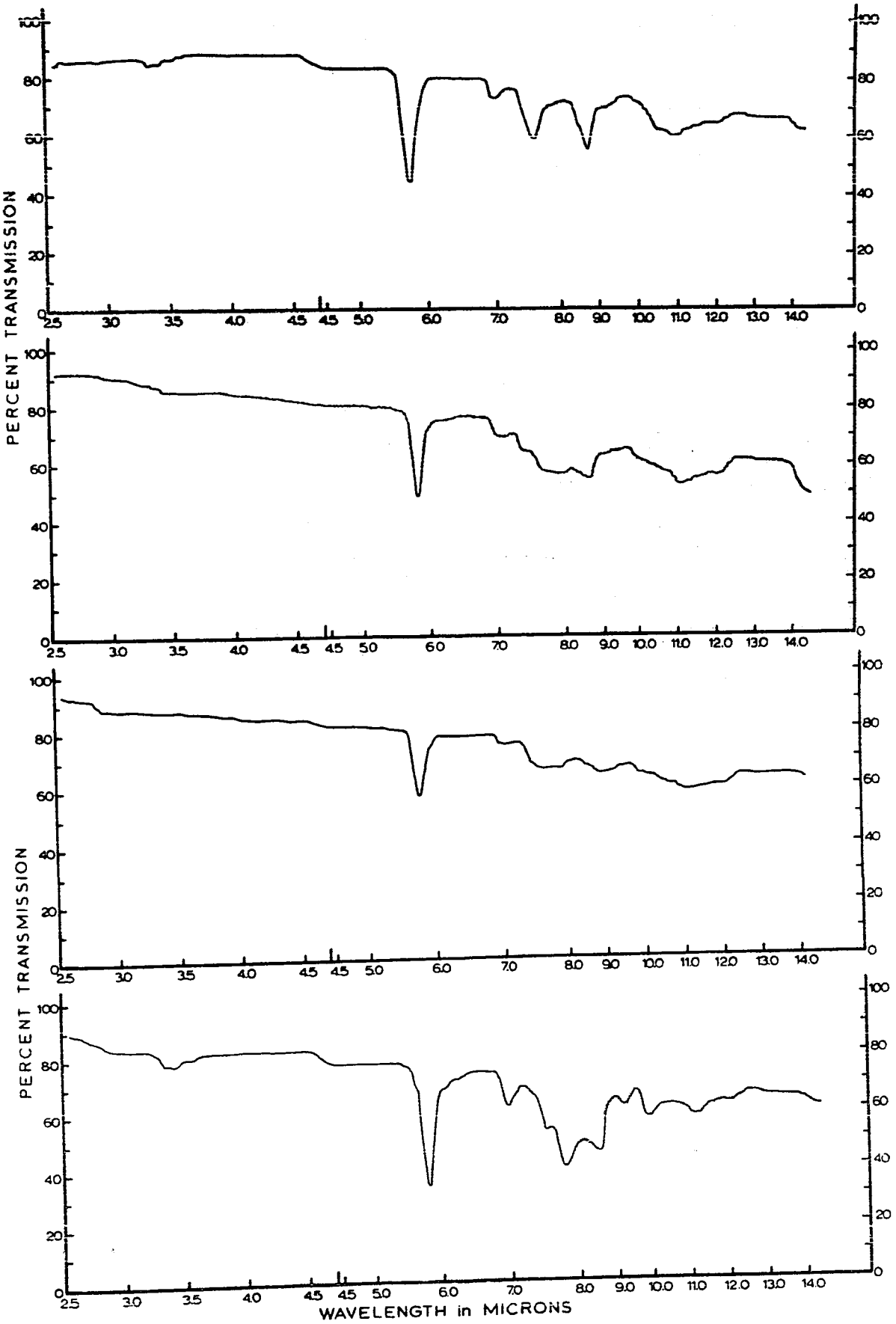
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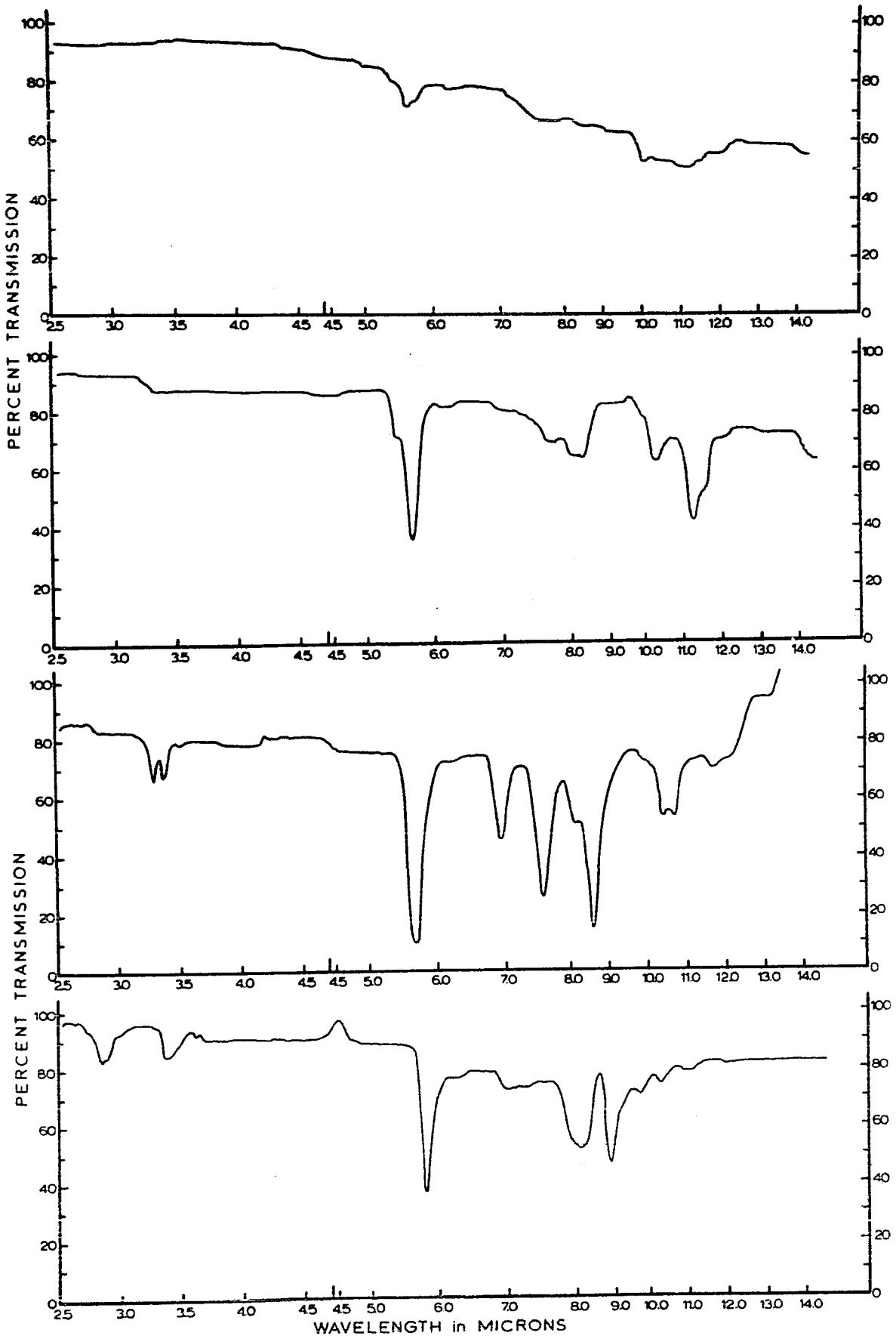
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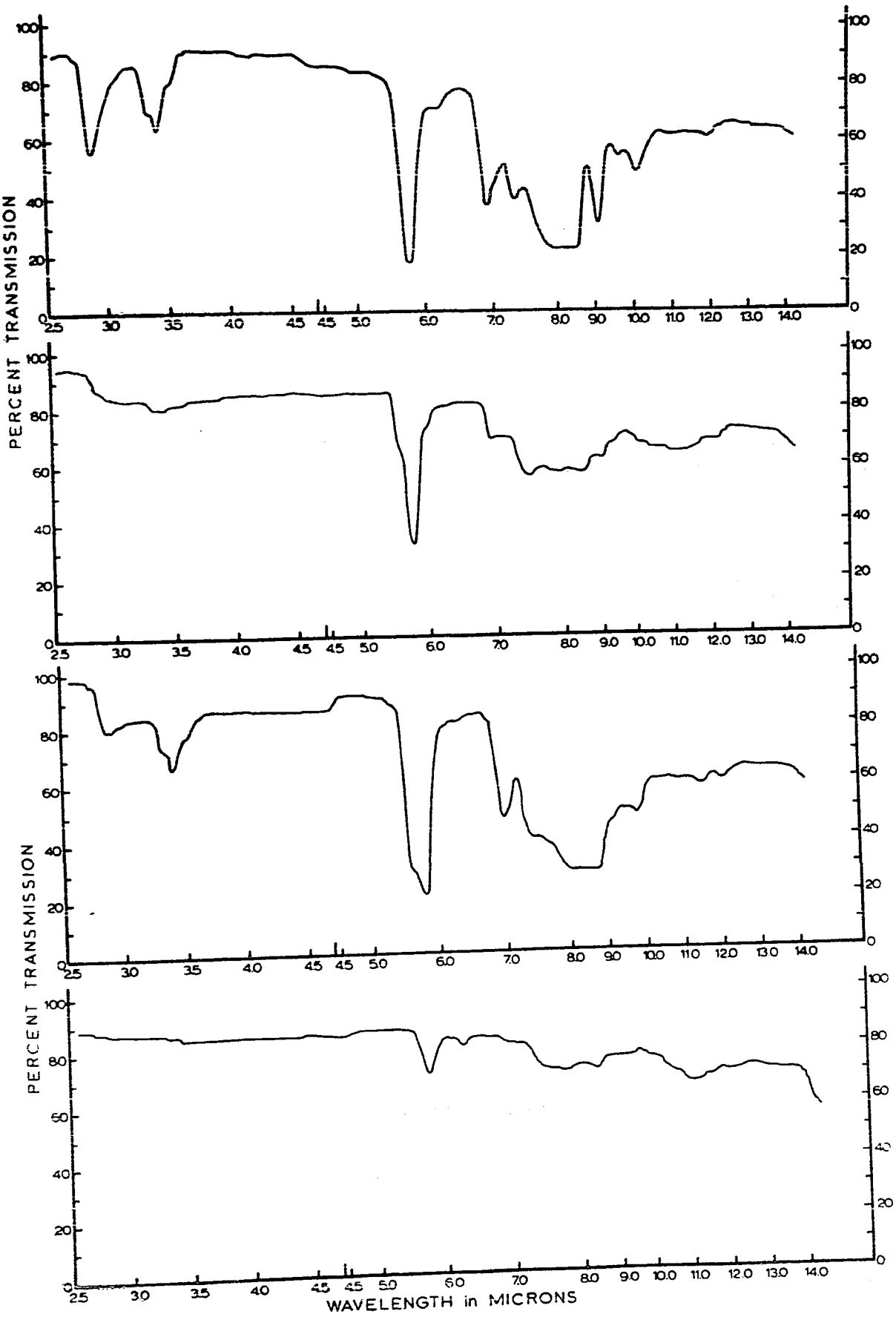
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CHAPTER IV

SUMMARY AND CONCLUSIONS

The organic acids of Bryophyllum calycinum leaf tissue were analyzed in order to identify or characterize the minor acids and determine the amounts of these acids. In addition to the major complement of malic, citric, and isocitric acids, this plant contains a high percentage of minor acids; the present work indicated that these acids comprise approximately 13% of the total acid content. The analysis of these acids is relevant since they may be involved in crassulacean acid metabolism and may be part of unknown metabolic pathways which result in the production and breakdown of acids.

These studies confirmed the presence of acids previously detected in B. calycinum and resulted in the identification or characterization of unreported acids. The minor acids identified in the leaf tissue were lactic, glyoxylic, oxalic, pyruvic, fumaric, succinic, oxalacetic, α-ketoglutaric, and cis-aconitic acids. In addition, a large number of acids were tentatively characterized but not identified. These were mono-, di-, or tricarboxylic acids apparently containing from three to seven carbons in the carbon chain. Many of these acids evidently had additional functional groups, including hydroxyl groups. The boiling points of the methyl esters of these acids ranged from about 140°C to 310°C. The wide variety of

structural types that these acids represent is in keeping with the different structures shown by known organic acids present in plant tissues.

The amounts of individual acids present in the leaf tissue were determined by gas chromatography of the methyl esters. Malic and isocitric acids each composed approximately 38% of the total acid content and the other major acid, citric acid, accounted for approximately 12%. Among the minor acids fumaric, pyruvic, and succinic acids each represented about 1% of the total acid content and cis-aconitic acid composed 1.5%. Small amounts of oxalic, lactic, and glyoxylic acids were determined but a portion of each of these acids may have been lost when the samples were dried prior to esterification. The unidentified minor acids were present in amounts ranging from less than 0.1% to 1.0%.

A major problem in these studies was the separation and determination of small amounts of acids in the presence of large amounts of major acids and numerous minor acids. One chromatographic method could not resolve completely all the acids, so chromatographic methods of analysis for organic acids were developed, modified, and combined in order to achieve separation of all the acids in the plant extract. The series of chromatographic procedures employed exploited different chemical characteristics of the acid molecules. The separation and determination of acids present in amounts ranging from 50% to 0.025% of the total acid content was possible using as little as 0.1 gram of dried leaf tissue. The methods employed would appear useful for the study of acids in other tissues, especially acids which are present in very small amounts.

The extraction of the acids from the dried leaf tissue with water containing H^+ form resin was apparently quantitative and gave reproducible results. The extract was free of cations and basic compounds, and the tissue and resin were easily removed by centrifugation and filtration.

The extracted acids could be conveniently separated on silica gel thin-layer plates. The use of acidic and basic solvents allowed the separation of acids which travel together in one solvent. Thin-layer chromatography involving this substrate was used for the analysis of acid fractions separated by other means and provided a valuable adjunct to these methods.

Separation of organic acids on cellulose layers 0.5 mm and 1.0 mm thick yielded good results with acidic and basic solvents. Acids could be quantitatively removed from the cellulose with 3N NH_4OH. Prewashing of the cellulose with NH_4OH solution was necessary to remove material which gave unwanted peaks during gas chromatography.

Sugars also apparently interfered with gas chromatographic analysis so these were removed by purifying the original plant extract on a carbonate form anion exchange column from which the acids were quantitatively recovered. The original acid extract could be fractionated by gradually increasing the concentration of the $(NH_4)_2CO_3$ eluant and this allowed a preliminary separation of major and minor acids.

Esterification of organic acids prior to gas chromatography was efficiently carried out with methanol containing H^+ form cation exchange resin. The esterification of organic acids was essentially

complete and esters could be conveniently and quantitatively recovered directly from the supernatant solution of the reaction mixture for use in gas chromatography. Low percent esterification and recovery of oxalic acid was encountered and oxalacetic acid gave only 80% esterification.

The polar stationary phase Carbowax 20 M allowed selective separation of the polar methyl esters by gas chromatography. The order of elution of the methyl esters from this column or a silicone column in general followed the order of the boiling points of the esters. Esterified pyruvic acid was eluted considerably later than expected and evidently a derivative with a high boiling point was formed during esterification. Three compounds were detected by gas chromatography after the esterification of oxalacetic acid and two after the esterification of α-ketoglutaric acid. The sensitivity of the keto acids was indicated by the formation of these compounds and has been a problem in previous methods of esterification. Isocitric acid was completely converted to dimethyl isocitric lactone during esterification and this resulted in a single peak for isocitric acid during gas chromatography. The peak was separated from that representing trimethyl citrate. Dimethyl malate and dimethyl tartrate, both of which contain a hydroxyl group, reacted with the Carbowax 20 M stationary phase.

The application of the above methods to the mixture of acids extracted from B. calycinum enabled the isolation of esters in a pure state suitable for infrared spectroscopy. As well, these methods allowed information to be obtained as to the characteristics and

identity of the acids when their behaviour was compared to that of known acids at all stages of separation. The preliminary fractionation of the organic acid extract on ion exchange resin provided information on the relative strength of the separated acids. Thin-layer or paper chromatography of the purified or original acid extract gave an indication of the number of carboxyl groups in the molecule. The presence of additional functional groups could also be predicted. The analysis by gas chromatography on silicone and Carbowax 20 M columns of acids esterified after recovery from paper or thin-layer chromatograms gave information as to the boiling points of the methyl esters of the acids. These boiling points indicated the number of carbons in the carbon chain and the number of carboxyl groups present in the acid. The separation on Carbowax 20 M when compared to that on silicone showed whether additional polar functional groups were present.

Infrared spectroscopy of the individual esters of plant acids, collected from the preparative silicone column, allowed identification of esters when their spectra were compared with those of known esters. Information as to the structure of unidentified esters could be obtained from the spectra. Esters which were prepared from known acids gave spectra which were similar but which could be distinguished, especially in the region greater than 7.0 microns. The small amounts of esters of plant acids available led to less distinct spectra but these were still sufficient for identification and characterization. The structure of characterized but unidentified esters could evidently be elucidated by comparing their spectra with those of esters of known acids showing similar chromatographic behaviour and having an apparently compatible structure.

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